MAP Kinases are Required for Apoptosis of HCT-8 Epithelial Cells Induced by the Parasite Giardia intestinalis

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Abstract

Giardia intestinalis is a protozoan that causes a generally self-limited clinical illness typically characterized by diarrhea, abdominal cramps, bloating, weight loss and malabsorption. The pathogenesis of giardiasis is multifactorial and probably different in various animal models, but the mechanisms responsible for the disease are still poorly understood. We previously reported that G. intestinalis is able to induce apoptosis in the human HCT-8 epithelial cell line through the activation of both the intrinsic and extrinsic apoptotic pathways. In the present study we demonstrate that activation of the mitogen-activated protein kinases (MAPKs) plays an important role in the regulation of HCT-8 cell apoptosis induced by G. intestinalis. MAPK activation seems to correlate with regulation of the apoptotic process because specific MAPK inhibitors significantly reduced the expression of the active form of caspase-3 in infected cells. Apoptotic changes were also dramatically inhibited by pre-treatment of the cells with JNK or p38 specific inhibitors, but not ERK 1/2 inhibitor. Taken together, these results suggest a critical role for MAPK activation in G. intestinalis-induced apoptosis in the human HCT-8 cell line.

Keywords: MAPK; Apoptosis; Giardia; HCT-8; Protozoa

Introduction

Giardia intestinalis (also known as G. lamblia and G. duodenalis) is a deeply divergent protist that causes intestinal infection worldwide. Infection can cause a generally self-limited clinical illness (i.e. giardiasis) typically characterized by diarrhea, abdominal cramps, bloating, weight loss and malabsorption, though asymptomatic infection frequently occurs [1-3]. Several reports have associated giardiasis with the development of chronic enteric disorders, allergies and reactive arthritis [4-7].

The parasite exists in two forms: infectious cysts and disease-causing trophozoites. In the gut lumen, trophozoites differentiate into cysts, which are passed in the host’s feces. Cysts have a filamentous wall that is resistant to harsh environmental conditions, facilitating the spread of giardiasis. For this reason, G. intestinalis poses a considerable problem, especially in the developing world where hygiene conditions are inadequate for blocking its transmission by the fecal-oral route.

Giardia species are ubiquitously distributed and have been detected in nearly all classes of vertebrates, including domestic animals and wildlife [8]. Recent advances in molecular epidemiology have led to the identification of specialized genetic groups (i.e., assemblages) that are relatively species-specific. Assemblages A and B of G. intestinalis primarily infect humans and primates, assemblages C and D infect dogs, assemblage F infects cats, assemblage E infects hoofstock and assemblage G infects rodents [9]. Although G. intestinalis infects both humans and animals, the role of zoonotic transmission to humans and the importance of animal contamination of food and water are being re-examined.

The pathogenesis of giardiasis is multifactorial and the mechanisms responsible for the disease are poorly understood [10]. We previously reported that G. intestinalis is able to induce apoptosis in the human HCT-8 epithelial cell line. In particular, we observed activation of both the intrinsic and extrinsic apoptotic pathways, down-regulation of the anti-apoptotic protein Bcl-2 and up-regulation of the pro-apoptotic Bax, suggesting a possible role of caspase-dependent apoptosis in the pathogenesis of giardiasis [11].

The mitogen-activated protein kinases (MAPKs), a family of related serine/threonine protein kinases, constitute a large kinase network that regulates a variety of physiological processes, such as cell growth, differentiation and apoptotic cell death [12]. The MAPKs family, comprising the extracellular signal regulated kinases (ERKs), c-Jun NH2-terminal kinase (JNK) and p38 MAPK, is of particular interest because its activation is involved in cell survival [13]. After MAPKs (ERK1, 2, JNK1–3 and p38) are activated either in the cytoplasm or in the nucleus, they bind and regulate transcription by modulating the function of a target transcription factor through serine/threonine phosphorylation [14]. In addition to the transcriptional effects of MAPKs signaling, accumulating evidence indicates that MAPKs regulate cellular behavior also by phosphorylating cytoplasmic target proteins, such as apoptotic proteins [12].

In order to decipher the mechanisms underlying programmed cell death (PCD) triggered by G. intestinalis infection of the intestinal cell, the molecules involved in cellular signaling were investigated. In particular, we investigated the role of MAPKs activation in the apoptosis of Giardia-infected cells.

Materials and Methods

Parasites

G. intestinalis strain WB clone 6 was used for this study.

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Trophozoites were grown axenically at 37°C in Keester’s modified TYI-S-33 medium [15], (pH 7.0) supplemented with bovine bile (0.52 mg/ml, Difco) and 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, all from Sigma-Aldrich (Milan, Italy).

**Cell cultures**

Cells of the human ileocecal adenocarcinoma line HCT-8 (ATCC CCL-244) were cultured, in controlled atmosphere (5% CO₂ atmosphere at 37°C) in RPMI 1640 medium (Sigma) supplemented with sodium pyruvate (1 mM), 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and L-glutamine (2 mM). Since duodenal and colonic epithelial cells are physiologically similar, cocultivation of *Giardia* trophozoites with colonic epithelial cells was chosen in order to investigate parasite-host cell interactions.

The mycoplasma infection status was routinely investigated in our cell cultures using a PCR Mycoplasma Detection Set (Takara, # 6601, Lonza, Milan, Italy) according to manufacturer’s instruction. Negative controls were included in every PCR experiment to ensure the absence of contamination.

**Infection of cells with *G. intestinalis* trophozoites**

Trophozoites were harvested by cold shock on ice for 30 min and centrifuged at 350 g for 10 min at 4°C. Then, after washes, live trophozoites were counted with a hemocytometer, added to cell cultures at a 3:1 (parasite/cell) ratio and incubated at 37°C in a 5% CO₂ atmosphere for different times (10 min-24 h) in order to establish the optimal time for observing MAPKs activation and cell apoptosis.

**Evaluation of apoptotic cells**

Apoptosis was morphologically assessed using the Vibrant Apoptosis YO-PRO assay (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s instructions in cell cultures subjected to infection with *G. intestinalis* trophozoites or treatment with the pro-apoptotic agent actinomycin D (5 mg/ml, Sigma). Untreated and uninfected cells were used as controls. Treated and untreated cells were incubated for 10 min to 24 h at 37°C. The percentage of apoptotic cells was compared to uninfected cells (Figure 1a).

**Molecular markers of apoptosis**

The bands obtained after immunoblotting were submitted to densitometric analysis using ID IMAGE ANALYSIS software (Kodak Digital Science). Results were expressed as relative optical density.

**SDS-PAGE and Western blotting**

**Densitometric analysis**

The bands obtained after immunoblotting were submitted to densitometric analysis using ID IMAGE ANALYSIS software (Kodak Digital Science). Results were expressed as relative optical density.

**Statistical analysis**

The data are presented as means ± SD. One-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test, was used to evaluate the differences among experimental data. A P value of < 0.05 was considered significant.

**Results**

**Molecular markers of apoptosis**

After 16 h of infection, cells exposed to *G. intestinalis* trophozoites exhibited the morphological features of apoptosis with characteristic membrane blebbing, chromatin condensation and pyknotic nuclei (data not shown). After staining with YO-PRO-1 dye and PI, apoptotic cells exhibited green fluorescence, dead cells exhibited red and green fluorescence and live cells showed little or no fluorescence (Figure 1a). The percentage of apoptotic cells was significantly (P < 0.001) higher after infection with *G. intestinalis* compared to uninfected cells (Figure 1b).

Activation of caspase-3 and PARP fragments, determined by Western blotting, was reported in our previous study, in which we observed that caspase-3 activation as well as PARP cleavage are activated in HCT-8 cells 16 h post-infection [11].
Involvement of MAPKs in *G. intestinalis*-induced apoptosis

We also investigated the expression of MAPKs in *G. intestinalis*-infected HCT-8 cells. We measured only slight phosphorylation of ERK 1/2 after *G. intestinalis* infection (Figure 2). The activation of ERK1/2 was significant (P < 0.05) after 20 min of incubation (Figure 2), but the phosphorylated form of ERK was no detectable after shorter (5 min and 10 min) or after longer incubation times (30 min to 24 h) (data not shown), suggesting early, but transient, activation. In addition, pretreatment with MEK inhibitor PD98059 almost completely inhibited *G. intestinalis*-induced activation. The phosphorylated form of ERK was detectable after 60 min of actinomycin incubation (data not shown), which is in agreement with observations in other studies [19].

High levels of the phosphorylated forms of p38 and JNK were observed after exposure to the viable trophozoites. Maximum levels of p38 and JNK activation were achieved after 60 min of incubation. Activation of p38 in response to *G. intestinalis* infection is shown in (Figure 3), it was clearly activated in infected HCT-8 cells but was barely detectable in control cells. On the contrary, the phosphorylation levels of p38 in *G. intestinalis* infected cells were not significantly different from those detectable in the controls after both shorter times of incubation (5 min and 10 min) and prolonged infection times (more than 60 min) (data not shown). Pre-treatment with SB203580 significantly inhibited *G. intestinalis*-induced phosphorylation of p38 MAPK.

JNK is a protein family of serine/threonine kinases encoded by three genes: JNK1, JNK2 and JNK3. The gene products can be alternatively spliced to yield alpha and beta protein forms. Thus, JNK1 and JNK2 can exist either as the full-length 54-kDa protein (p54) or as a COOH-terminally truncated 46-kDa form (p46). JNK1 and JNK2 are ubiquitously expressed, including in enterocytes, whereas JNK3 is present in the nervous system and cardiomyocytes but not expressed in the small intestine [18]. We determined the levels of immunoreactive phospho-JNK (p-JNK) in lysates of intestinal cells using Western blot and found the activated JNK form (p46) after 60 min of infection with *G. intestinalis* (Figure 4). As well as for the p38, the levels of phosphorylated form of JNK in *G. intestinalis* infected cells were not significantly different from those detectable in the controls after both shorter times of incubation (5 min and 10 min) and prolonged infection times (more than 60 min) (data not shown). Densitometric analysis revealed that the p46 form of p-JNK was significantly increased at 60 min (P < 0.001). In addition, pre-treatment with the specific inhibitor SP600125 almost completely inhibited *G. intestinalis*-induced activation.

Low levels of the phosphorylated forms of MAPKs (ERK1/2, p38 and JNK) were observed in uninfected HCT-8 cells (Figures 2-4). In order to investigate whether the activation of MAPKs is associated with *HCT* apoptosis induced by 16 h *G. intestinalis* infection, we evaluated the inhibitory effect of specific MAPK inhibitors PD98059, SP600125 and SB203580 (which inhibit ERK1/2, JNK and p38, respectively) on the percentage of apoptotic cells. As shown in (Figure...
5), the ERK1/2 inhibitor, PD98059, did not exhibit any inhibitory effect on *G. intestinalis*-induced apoptosis, whereas SP600125 and SB203580 significantly reduced HCT-8 apoptosis compared to control. Interestingly, when SP600125 and SB203580 were simultaneously added to cell cultures, apoptosis was almost completely suppressed, suggesting the involvement of both p38 MAPK and JNK in the apoptotic process (Figure 5).

Finally, we examined the role of MAPKs in caspase-3 activation following 16 h incubation with *G. intestinalis*. We observed that pre-treatment with the different inhibitors significantly reduced ($P < 0.001$) caspase-3 expression in HTC-8 cells. Though the ERK1/2 inhibitor weakly reduced the levels of caspase-3, both SP600125 and SB203580 were able to strongly reduce the expression of the cleaved form of caspase-3 and to block this expression when simultaneously added to cells (Figure 6). These results suggest that p38 MAPK and JNK are involved in the activation of intracellular caspase-3 in *G. intestinalis*-infected cells.

**Discussion**

Apoptosis can be induced by a variety of different signals, including activation of Fas or tumor necrosis factor receptors, deprivation of growth factors and different stresses and is mediated by the caspase enzymes. Several pathogens have been reported to trigger apoptosis or modulate apoptosis by interfering with the cell survival pathways. Here, we report that *G. intestinalis* induces apoptosis through the activation of caspase-3 in the human intestinal cell line HCT-8. The present study demonstrates that MAPK activation plays an important role in the regulation of apoptosis induced by *G. intestinalis*. Incubation of HCT-8 cells with viable trophozoites of *G. intestinalis* resulted in a significant increase in MAPK activation. In particular, ERK was barely activated in infected cells. Finally, we demonstrated that apoptotic changes were dramatically inhibited by pre-treating the cells with both the JNK and p38 specific inhibitors, but not the ERK1/2 inhibitor. Taken together, these results suggest a critical role for MAPK activation in *G. intestinalis*-induced apoptosis in HCT-8 cells.

Apoptosis is well known to be under tight regulatory control. Among these regulators, MAPKs are important mediators of apoptosis in all eukaryotic organisms [19]. In mammalian cells, at least three MAPKs subtypes are known, namely ERK1/2, p38, and JNK. The ERK cascade is mainly activated through receptor-mediated signaling stimuli, including growth factors and is associated with cell proliferation, differentiation and contributes to some cases of cell death.

**Figure 4:** Immunoblotting and densitometric analysis of phosphorylated (pJNK1/2) and total unphosphorylated (JNK1/2) protein in HCT-8 cells infected by *G. intestinalis* after 60 min of incubation. CTR, control cells; G, *G. intestinalis*-infected cells; G+SP600125, after pre-treatment with the specific inhibitor of JNK1/2; ACT, actinomycin D-treated cells. Results are expressed as means ± SD of five different experiments. *$P < 0.05$ compared to CTR, **$P < 0.01$ compared to CTR.

**Figure 5:** Involvement of MAPKs in HCT-8 cell apoptosis induced by *G. intestinalis* after 16 h of incubation. Percentage of apoptosis in unstimulated cells (CTR), actinomycin D-treated cells (ACT), *G. intestinalis*-infected cells (G), and *G. intestinalis*-infected cells after pre-treatment with PD98059, SB203580, SP600125, or SB203580 + SP600125. Results are expressed as means ± SD of five independent experiments. **$P < 0.01$ compared to G.

**Figure 6:** Involvement of MAPKs in caspase-3 activation by *G. intestinalis* after 16 h of incubation. Immunoblotting and densitometric analysis of caspase-3 activation in unstimulated cells (CTR), actinomycin D-treated cells (ACT), *G. intestinalis*-infected cells (G), and *G. intestinalis*-infected cells after pre-treatment with PD98059, SB203580, SP600125, or SB203580 + SP600125. Results are expressed as means ± SD of five independent experiments. **$P < 0.01$ compared to G.
[20,21]. In contrast, p38 and JNK (also called stress-activated protein kinase, SAPK) cascades are activated in response to treatment with pro-inflammatory or stressful stimuli, including bacterial and viral infection and appear to be closely related to cell death [22-25]. Apoptosis is an important factor in both parasite survival and pathogenicity [26-28], exhibiting multifaceted roles in host-parasite interactions. In regards to *Giardia* infection, previous studies have indicated a relationship between enterocyte apoptosis and loss of the epithelial barrier function in a human duodenal epithelial cell line infected with trophozoites of certain strains of *G. lamblia* [29], though the mechanisms underlying this process are still under investigation.

Several reports in the literature suggest a pivotal role for MAPKs in the regulation of apoptosis by intracellular parasites. For example, the p38 MAPK signaling cascade is a requisite for the apoptosis of *Trichomonas vaginalis*-infected macrophages, via the phosphorylation of p38 MAPK [30]. *Yersinia enterocolitica* down-regulates JNK, p38 MAPK and ERK1/2 in macrophages. Moreover, evidence suggests that *Entamoeba histolytica* induces apoptosis of host cells, including neutrophils, T lymphocytes and macrophages and that ERK1/2 activation is required for this process [31].

Other evidence has shown that infection with the promastigote forms of *Leishmania major* and *Leishmania donovani* induces infected cells to resist apoptosis [27,32-34] and that survival of the intracellular protozoan correlates with attenuation of MAPK signaling, though the role for kinases in the apoptotic program is controversial. In fact, several investigators have reported that signaling through p38 MAPK, like other signaling pathways, is either avoided by *Leishmania* parasites or actively suppressed [35]. Nonetheless, at least two reports have demonstrated the appearance of phosphorylated p38 MAPK soon after infection with *Leishmania* [36,37]. Finally, p38 MAPK and JNK signaling cascades have also been shown to be involved in apoptosis induced by Reoviridae family members, including HIV-1, herpes simplex virus 1 and hepatitis C virus. The molecular basis for these different observations is still unknown.

Therefore, the involvement of ERK1/2, JNK and p38 MAPK during apoptosis regulation is dependent upon different kinds of stimuli and cell types. The MAPK-mediated signaling pathways demonstrate that activation of JNK and p38 MAPK with concurrent inhibition of ERK is critical to the induction of apoptosis [25]. Therefore, the dynamic activation of JNK and p38 (Figure 7). The present study offers important insights that may help us gain a better understanding of the molecular mechanisms involved in *Giardia*-associated pathogenesis.

**Conclusions**

In summary, the results of the present study provide the first evidence that live trophozoites of *G. intestinalis* are able to induce apoptosis in HCT-8 cells through the activation of MAPKs JNK and p38 (Figure 7). The present study offers important insights that may help us gain a better understanding of the molecular mechanisms involved in *Giardia*-associated pathogenesis.

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