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Anoikis potential of *Entameba histolytica* secretory cysteine proteases: Evidence of contact independent host cell death

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ABSTRACT

Mammalian epithelial, endothelial and various other cell types, upon their detachment from the extracellular matrix (ECM) undergo a specialized kind of apoptosis, known as anoikis. *Entameba histolytica* cysteine proteases have been implicated in degradation of the host ECM, which may induce anoikis in host cells. To explore this hypothesis, supernatant obtained from 2 h in-vitro cultivation of *E. histolytica* (SRP), was used as a source of cysteine proteases. MDA-MB-231 (human mammary epithelial adenocarcinoma) cells were treated with SRP and their detachment and apoptosis was evaluated. 25 μg/ml (with respect to protein concentration), SRP was found to be the optimal concentration to dislodge over 98% MDA-MB-231 cells from monolayer in 20 min. The detachment was followed by apoptosis of at least 41.2% cells, characterized by caspase-3 dependent inter-nucleosomal DNA fragmentation. The SRP-induced apoptosis was associated exclusively with the detached fraction. Moreover, detachment preceded apoptosis. E-64 (a cysteine protease inhibitor) abolished the SRP-induced detachment as well as inter-nucleosomal DNA fragmentation. Interestingly, SRP induced a 3.21 fold increase in the JNK activity, whilst SP600125 (a JNK inhibitor) blocked the SRP-induced inter-nucleosomal DNA fragmentation. Thus, it was concluded that spontaneously released cysteine proteases of *E. histolytica* can induce JNK dependent anoikis of MDA-MB-231 cells, which may be implicated in contact independent host cell death during amebiasis.

1. Introduction

Amebiasis is one of the most aggressive and is considered to be the third leading cause of death amongst parasitic diseases, surpassed only by malaria and Schistosomiasis [1]. Invasive amebiasis is responsible for enteric syndromes ranging from dysentery to colitis, which is highly lethal. Sometimes, active trophozoites invade the portal circulation, through the bowl wall and disseminate systemically, reaching the liver to cause hepatic amebiasis. Following the depression of the protective mucus blanket, trophozoites attach to the cells of the inter-glandular epithelium and with the aid of proteolytic enzymes, especially cysteine proteases, degrade elastin, collagen and fibronectin; the components of extracellular matrix (ECM). This invasion is followed by lateral extension of the destructive lesions in the sub-mucosal layer, creating classical flask shaped ulcers [reviewed in [2–4]]. Despite various studies on pathogenesis of amebiasis, the mechanism of cell death at the site of invasion remains illusive.

Earlier investigations provide evidence of apoptotic death of host cells by *Entameba histolytica* [5–8]. However, most of them show that the apoptosis of the host cells depends on contact with the Entameba trophozoites [5,7,9]. Interestingly, all the studies showing contact-dependent apoptosis were conducted in myeloid or lymphoid cell types [9–11]. As per the current knowledge about mammalian cell death, the mechanism of cell death varies with cell type. Therefore, the contact-dependency of the death of the myeloid/lymphoid cells induced by *E. histolytica* may not be applicable to the other cell types, particularly intestinal and hepatic cells, the most commonly affected organs in invasive amebiasis. Hepatocyte and enterocyte apoptosis by *E. histolytica* is very well documented [6,12,13]. Further, the hepatocyte apoptosis has been implicated in amebic liver abscess (ALA). Interestingly, amebic cysteine proteases have been implicated in ALA too [12]. So apparently it seems that there is a link between cysteine protease mediated tissue damage, host-cell apoptosis and amebic liver abscess and/or colonic ulcer formation.

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The intestinal epithelial cells grow in an adhesion dependent manner, where they require attachment with ECM for their growth and survival [14]. The degradation of ECM-Cell attachment causes a specialized type of apoptosis, known as anoikis [15]. ECM degradation is a well known phenomenon in amebiasis [16], which may render cells devoid of contact with the ECM. So, it was hypothesized that anoikis may be involved in amebiasis. Therefore, the major objective of the present investigation was to explore the anoikis potential of *E. histolytica* cysteine proteases. As the cysteine proteases are known to be secreted spontaneously during in-vitro cultivation of *E. histolytica* [17], the supernatant of 2 h in-vitro culture of *E. histolytica* was used as a source of cytsteine protease. On the other hand, the MDA-MB-231 (human mammary epithelial cell adenocrcinoma) was used as anoikis susceptible cell line [18,19] while Jurkat (which grows in adhesion independent manner) was used as an anoikis resistant cell line.

2. Results

2.1. SRP causes detachment of MDA-MB-231 monolayer

To evaluate the morphological changes of the MDA-MB-231 monolayer inflicted by SRP, the monolayer was treated with different doses of SRP for various time points and was photographed with a digital camera mounted on an inverted microscope. The detachment effect was found to be dose dependent. Upon 20 min incubation with SRP, 5 μg or 10 μg resulted in detachment of <10% cells whereas 30% and 98% cells were detached with 20 μg, and 25 μg respectively (Fig. 1A and B). Similarly the duration of treatment also affected the detachment of cells. At 20 min of treatment with 25 μg/ml of SRP >98% cells were detached, whereas 18% and 56% cells were detached at 5 min and 15 min respectively (Fig. 1A and B).

2.2. SRP induces apoptosis of adhesion dependent MDA-MB-231 but not of Jurkat

Since adhesion dependent cells undergo apoptosis after detachment, we wanted to explore the fate of the cells which show detachment by SRP treatment. Therefore, the MDA-MB-231 monolayer was treated with 25 μg SRP for indicated time (Fig. 2A), after which all the cells (attached and detached) were pooled and their DNA was isolated. The DNA from each sample was separated on 1.5% agarose gel. Intenucleosomal DNA fragments were seen in samples treated with SRP for 8 h or more. The maximum inter-nucleosomal DNA fragmentation was seen at 24 h of SRP treatment, which did not increase further until 36 h of treatment (Fig. 2A). Caspase-3 activity was found to be significantly higher in SRP treated samples compared to control after 6 h of incubation. However, no significant increase in caspase-3 activity was found at or before 4 h of treatment (Fig. 2B). Since the cellular detachment is complete within 20 min, this observation shows that there is a delay between detachment and augmentation of apoptosis. This correlates well with the fact that DNA degradation occurred only after 8 h of SRP treatment (Fig. 2B). The cell cycle analysis revealed that until 8 h of SRP treatment there is a slight increase in the sub-G0/G1 population (3.53%) compared to control (0.41%), whereas there is a significant increase in the same at 12 h (14.2%) and 24 h (41.27%) (Fig. 2C). The, z-VAD-FMK (a pan caspase inhibitor) completely abolished the SRP-induced inter-nucleosomal DNA fragmentation (Fig. 2D). On the other hand, Jurkat cells treated with SRP in a similar way to the MDA-MB-231 cell line did not show any inter-nucleosomal DNA fragmentation, even after 36 h of treatment (Fig. 2F).

To know whether the SRP-induced inter-nucleosomal DNA fragmentation was associated with the detached or attached cells, a suboptimal dose (5 μg) of SRP was selected, which detached only 40% cells of the monolayer, upon 24 h treatment. The attached and detached fractions were subjected to the DNA laddering assay, which revealed inter-nucleosomal DNA fragments in the detached fraction while there was none in the attached fraction (Fig. 2E).

2.3. Re-seeding of SRP treated MDA-MB-231

Previous experiments demonstrated that apoptosis is associated with detached cells. Because the cells undergoing apoptosis also exhibit detachment, we wanted to know whether the detachment induced by SRP is the cause or the effect of apoptosis, i.e. whether detachment is an upstream or downstream event to apoptosis. If detached living cells are reseeded, they are expected to exhibit no signs of apoptosis. Therefore, MDA-MB-231 cells were reseeded after incubation with SRP for various times. Inter-nucleosomal DNA fragmentation assay occurs only after 8 h of SRP treatment and is maximal at 24 h (Fig. 2A). Therefore, inter-nucleosomal DNA fragmentation was evaluated after 24 h of SRP treatment. Samples that were treated with SRP for less than 1 h before reseeding did not show any inter-nucleosomal DNA fragments. On the other hand, samples that were treated with SRP for 2 h or more showed inter-nucleosomal DNA fragmentation (Fig. 3).

Interestingly, cells reseeded after 15–60 min of SRP treatment completely attached to the surface of the tissue culture flask (data not shown), which would be expected from the living cells. However, the cells remain afloat in media for 2–3 h before they attached completely (data not shown). Thus, the total time for which the cells remained under suspension was 15 min + 2–3 h, 30 min + 2–3 h and likewise. In this experiment, the cells required at least 2 h of SRP treatment for apoptosis (Fig. 3), thereby staying under suspension for at least 4–5 h before undergoing apoptosis.

2.4. Involvement of cysteine proteases in SRP-induced apoptosis

In this investigation detachment was found to be involved in SRP-induced apoptosis. Since cysteine proteases are secreted during the in-vitro cultivation of Entameba [17], which have been implicated in monolayer detachment [20], we evaluated the involvement of cysteine proteases in SRP-induced detachment and apoptosis. The MDA-MB-231 monolayer was treated with SRP (25 μg/ml) in the presence or absence of E-64 (a cysteine protease inhibitor). E-64 + SRP treated samples showed normal monolayer whereas SRP treated samples showed detached cells within 5–20 min of SRP treatment (Fig. 4A). Moreover, E-64 + SRP treated samples showed no inter-nucleosomal DNA fragmentation, which was clearly observed in SRP treated samples (Fig. 4B). Further, the cell cycle analysis showed 5% apoptotic cells in samples treated with E-64 + SRP, whereas 38% apoptotic cells were associated with the SRP treated samples (Fig. 4C).

2.5. Involvement of JNK in SRP-induced apoptosis

JNK activity has been implicated in detachment and anoikis [21]. Since we had found the SRP induced apoptosis involved detachment, we wanted to elucidate whether JNK activity is involved in SRP-induced apoptosis. The active JNK phosphorylates c-Jun, thereby increasing the phospho-c-Jun level. SRP treated samples showed 3 fold more phospho-c-Jun compared to control (without SRP treatment) (Fig. 5A). Moreover, SRP + SP600125 (a JNK inhibitor) treated samples showed no inter-nucleosomal DNA fragments (Fig. 5B). The cell cycle analysis showed 8% apoptotic cells associated with SRP + SP600125 treated samples whereas 33% apoptotic cells associated with control (only SRP treated samples) (Fig. 5C).