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Ultrastructural observation of human neutrophils during apoptotic cell death triggered by *Entamoeba histolytica*

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Abstract: Neutrophils are important effector cells against protozoan extracellular parasite *Entamoeba histolytica*, which causes amoebic colitis and liver abscess in human beings. Apoptotic cell death of neutrophils is an important event in the resolution of inflammation and parasite's survival in vivo. This study was undertaken to investigate the ultrastructural aspects of apoptotic cells during neutrophil death triggered by *Entamoeba histolytica*. Isolated human neutrophils from the peripheral blood were incubated with or without live trophozoites of *E. histolytica* and examined by transmission electron microscopy (TEM). Neutrophils incubated with *E. histolytica* were observed to show apoptotic characteristics, such as compaction of the nuclear chromatin and swelling of the nuclear envelop. In contrast, neutrophils incubated in the absence of the amoeba had many protrusions of irregular cell surfaces and heterogenous nuclear chromatin. Therefore, it is suggested that *Entamoeba*-induced neutrophil apoptosis contribute to prevent unwanted tissue inflammation and damage in the amoeba-invaded lesions in vivo.

Key words: *Entamoeba histolytica*, neutrophils, transmission electron microscopy, apoptosis, inflammation

Entamoeba histolytica causes amoebic dysentery and amoebic liver abscesses (Stanley, 2003). Fifty million cases of invasive amebiasis and 100,000 deaths worldwide are estimated to occur annually (Petri et al., 2002). Upon invasion, it may lyse various host cells including colonic epithelial cells, endothelial cells, and also cellular effector cells of the defense system (Berninghausen and Leippe, 1997). Recently, it has also been demonstrated that *E. histolytica*-induced host cell apoptosis occurs through caspase-dependent mechanism (Huston et al., 2000). Neutrophils are the short-lived primary effector cells in host defense against injury and infection, and the activation of neutrophils is an important amoebicidal factor (Seydel et al., 1997; Ghosh et al., 2000; Jarilo-Luna et al., 2002). Upon activation, neutrophils secrete a variety of molecules, such as reactive oxygen species and proteolytic enzymes, which can kill invading microorganisms, and cause substantial local tissue damage (Takazoe K et al., 2000). Thus, neutrophil apoptosis plays a key role in the resolution of inflammation (Savill, 1997). The typical morphological features of apoptosis include cell shrinkage, budding, DNA degradation to

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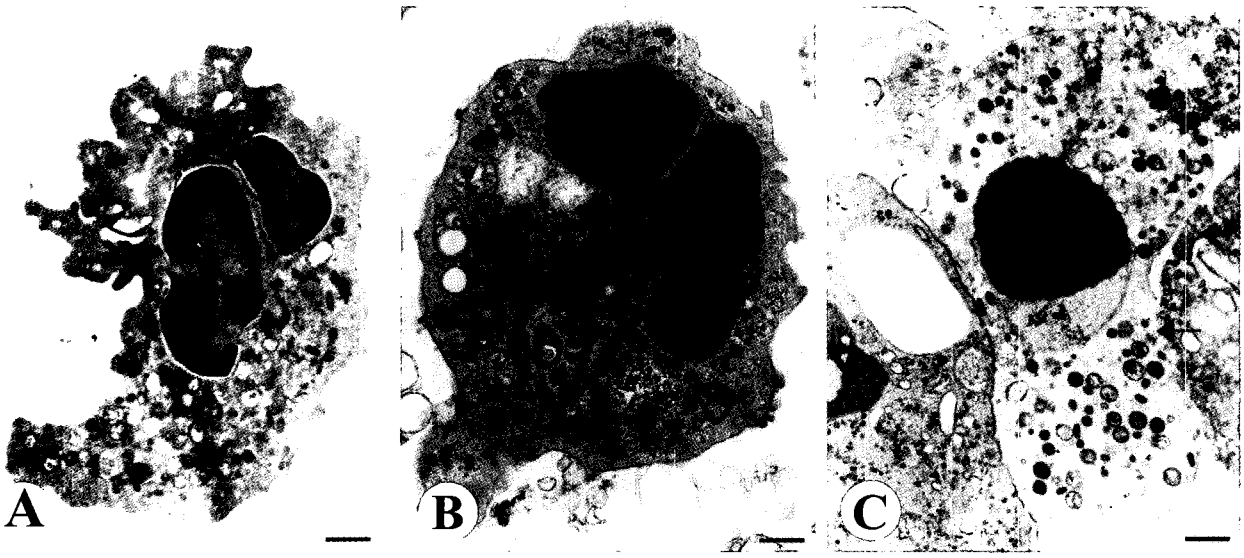

Fig. 1. TEM view of apoptotic neutrophils induced by *Entamoeba histolytica*. Freshly isolated neutrophils (2×10^6/well) were incubated for 15, 30, or 60 min at 37°C with or without *E. histolytica* (2×10^5/well) and then processed for TEM as described in the text. X 14,000. **A.** Neutrophils incubated for 30 min with medium alone contained nuclear lobes filled with heterochromatin. The cytoplasm was typical of normal neutrophil with numerous granules, mitochondria, and granular cytoplasmic matrix. **B.** Neutrophils incubated for 15 min with *E. histolytica* showed loss of irregular surface margins, and smooth outlines. Nuclei contained both eu- and hetero-chromatin. **C.** Neutrophils incubated for 30 min with *E. histolytica* contained a round nucleus filled with condensed chromatin, and the perinuclear space had widened (arrow). Bar = 1 μm.

form a characteristic ladder when analyzed by electrophoresis, and its ultrastructural characteristics include chromatin condensation, shrinkage and fragmentation of nuclei, the formation of micronuclei and apoptotic bodies, condensation of cytoplasm, and blebs from the cell surface (Vaux, 1993; Vermes and Haanen, 1994; Guejes et al., 2003). However, the ultrastructural changes or features of apoptotic neutrophils during cell death caused by *E. histolytica* have not been previously investigated.

E. histolytica trophozoites (strain HM1:IMSS) were maintained axenically in TYI-S-33 medium at 37°C. During the late logarithmic phase, trophozoites were harvested by centrifugation at 200 *g* at 4°C for 5 min after being chilled in an ice bath for 10 min and suspended in RPMI 1640 culture medium supplemented with $NaHCO_3$ 2 g/L, gentamycin 50 mg/L, human serum albumin 1 g/L and 10% (v/v) heat-inactivated FCS. Peripheral blood neutrophils were isolated from healthy donors. Briefly, heparinized blood was collected and diluted with piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES) buffer containing 25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, and 5.4 mM glucose (pH 7.4) at a 1:1 ratio. The diluted blood was then carefully overlaid on Histopaque (Histopaque 1083; Sigma Chemical Company) and centrifuged at 1,000 *g* at 4°C for 30 min. Isolated fractions were cleared of erythrocytes by fast lysis with ice-cold distilled water. Neutrophils isolated by this procedure were more than 95% pure. Immediately following the isolation procedure, neutrophils (2×10^6/well) were incubated with culture medium at 37°C for 15, 30 or 60 min in a humidified CO_2 incubator (5% CO_2, 95% air) with or without *E. histolytica* trophozoites (2×10^5/well) in 24 well tissue culture plates. After washing with PBS, portions of neutrophil sediments were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight and then washed with the same buffer. They were then post-fixed in 1% osmium tetroxide in the same buffer at 4°C for 1 hr and dehydrated. After dehydration, the cells were embedded in Epon 812, ultrathin sections

were prepared by using an LKB-V ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (JEOL 100CX-II).

Transmission electron microscopy (TEM) revealed the ultrastructural alterations of cells undergoing apoptosis in human neutrophils incubated with *E. histolytica*. Healthy neutrophils have irregular cell surfaces, polysegmented nuclei, and heterogenous nuclear chromatin without swelling of the nuclear envelope (Fig. 1A). Following incubation with *E. histolytica* for 15 min, the shapes of neutrophils became round with slight condensation of chromatin and cytoplasm (Fig. 1B). Furthermore, after 30 min of incubation, neutrophils in close contact with a trophozoites showed apoptotic appearance, as evidenced by marked condensation of nuclear chromatin and swelling of the nuclear envelope (Fig. 1C). After 60 min of incubation with *E. histolytica*, neutrophils were found be necrotic morphology such as disruption of plasma membranes, cytoplasmic vacuolation, and loss of cellular contents (data not shown).

It has been known that the majority of dying cells after contact with *E. histolytica* show a combination of apoptotic and necrotic morphology in vitro (Ragland et al., 1994), although only a small percentage of cells had a necrotic or apoptotic appearance exclusively. Apoptotic cell death of human neutrophils induced by *E. histolytica* is considered to be a important mechanism to lessen neutrophil-mediated tissue inflammation and damage in the parasite-infected lesions, because apoptotic cells can then be recognized and phagocytosed by macrophages in a non-phlogistic manner: that is, there is no release of pro-inflammatory mediators but a release of potential anti-inflammatory mediators such as transforming growth factor β1 (TGF-β1) and interleukin 10 (IL-10) (Simon, 2003). Therefore, these observation led us to support universal finding that amoebae cause little or minimal inflammatory responses despite of extensive tissue lytic necrosis during liver invasion of *E. histolytica*. In contrast, when cells become necrotic they have the potential to cause tissue injury and because of the release of proinflammatory mediators will amplify the inflammatory process. Following phagocytosis of necrotic cells, macrophages liberate pro-inflammatory mediators such as thromboxane B_2 (TxB_2), IL-8 and tumour necrosis factor (TNF-α). Therefore, it should be investigated how *E. histolytica* can induce apoptosis and/or necrosis in immune cells to unravel complex milieu of inflammation in the parasite-infected lesions during amoebiasis in human beings.

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