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Trichomonas vaginalis promotes apoptosis of human neutrophils by activating caspase-3 and reducing Mcl-1 expression

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SUMMARY

Neutrophils are the predominant inflammatory cells found in the vaginal discharge of patients with Trichomonas vaginalis *infection. However, it is not known whether neutrophil apoptosis is induced by live* T. vaginalis. *Therefore, we examined whether* T. vaginalis *can influence neutrophil apoptosis, and also whether caspase-3 and the Bcl-2 family members are involved in the apoptosis. Thus, human neutrophils were incubated with live* T. vaginalis *and neutrophil apoptosis was evaluated by Giemsa, annexin V-PI, and $DiOC_6$ stainings. The neutrophil apoptosis was significantly higher in those incubated with* T. vaginalis *than in the control group. When trichomonads were pre-treated with mAb to AP65 (adhesin protein), or when trophozoites were separated from neutrophils using a Transwell chamber, neutrophil apoptosis was significantly reduced. The activation of caspase-3 was evident in neutrophils undergoing spontaneous apoptosis but was markedly enhanced during* T. vaginalis*-induced apoptosis. Moreover, the inhibition of caspase-3 effectively reduced* T. vaginalis*-induced apoptosis. Trichomonad-induced apoptosis was also associated with reduced expression of the neutrophil anti-apoptotic protein, Mcl-1. These results indicate that* T. vaginalis *alters Mcl-1 expression and caspase-3 activation, thereby inducing apoptosis of human neutrophils.*

Keywords Trichomonas vaginalis*, neutrophil, apoptosis, caspase-3, Mcl-1*

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INTRODUCTION

Trichomonas vaginalis commonly causes vaginitis and perhaps cervicitis in women and urethritis in both sexes (1). In pregnant women, trichomonads are implicated in the premature rupture of membranes, premature delivery, and the delivery of low birth weight infants (2,3). In addition, trichomoniasis has been implicated as a risk factor for human immunodeficiency virus (HIV) transmission (4,5). More than 173 million people worldwide are infected annually by this parasite (6) and its prevalence rate in Kuri City, Korea, was reported to be 10·4% (7).

Although *T. vaginalis* is known to be a non-invasive microorganism, it elicits the vaginal mucosal infiltration of inflammatory cells, especially neutrophils. As proof of neutrophil infiltration, Rein *et al*. (8) reported a polymorphonuclear leucocytes (PMN)/epithelial cell ratio > 1 in 47 of 65 (72%) women with trichomoniasis by wet mount, and 15% of these women had PMN/epithelial cell ratios > 10. Also, PMN fused with trichomonads have been detected in vaginal smears of trichomoniasis by microscopic observations (9).

Only a few studies have been carried out on the response of neutrophils to *T. vaginalis* infection. *T. vaginalis*-induced recruitment of neutrophils is known to be mediated via the IL-8 expressed by neutrophils in response to activation by live *T. vaginalis* (10), although the role of infiltrated neutrophils in the pathogenesis of *T. vaginalis* infection has not yet been clearly characterized.

Neutrophils have a shorter life span than other leucocytes. After egress from the bone marrow, neutrophils leave the circulation within 6–10 h and migrate into tissues, where they undergo constitutive apoptosis in 1–2 days. However, the life span of neutrophils can be either prolonged or shortened by signals from the micro-environment (11). Various inflammatory mediators such as cytokines and bacterial products, and local conditions such as hypoxia and the expression of Fas/Fas ligand, are known to promote or suppress neutrophil apoptosis at the site of infection, thus regulating the

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progression of inflammatory responses (12). However, no report to date has described neutrophil apoptosis after *T. vaginalis* stimulation.

Recent studies implicate the involvement of caspases and members of the Bcl-2 protein family in the regulation and execution of neutrophil apoptosis (13). The caspases, a family of cysteine proteases, are activated by a set of pro-apoptotic signals (14). Caspase-3, also called executioner caspase, recognizes and cleaves the sequence Asp-Glu-Val-Asp (DEVD) in poly-ADP-ribose polymerase (PARP), resulting in irreversible nuclear alteration (15). In fact, a caspase-3 null cell line does not exhibit DNA fragmentation or some of the distinct morphological features typical of apoptotic cells such as shrinkage or blebbing (16). The Bcl-2 protein family constitutes an important intracellular checkpoint of apoptosis within a common cell death pathway. Recently, multiple Bcl-2 homologues have been identified as either pro-apoptotic or anti-apoptotic checkpoints upstream of the caspases (17–19). Moreover, the balance between pro-apoptotic and anti-apoptotic pathways determines the fate of cells in many systems.

In this study, to elucidate the role of neutrophils in vaginal inflammation caused by *T. vaginalis* infection, we examined neutrophil apoptosis induced by live *T. vaginalis* and evaluated the role of regulatory factors such as caspase-3 and Bcl-2 protein family, which are involved in neutrophil apoptosis. We demonstrate that co-incubation with *T. vaginalis* results in the apoptosis of neutrophils, and that this is associated with an increase of caspase-3 activity and Mcl-1 protein levels.

MATERIALS AND METHODS

Reagents

Cycloheximide, PIPES [piperazine-N,N′-bis(2-ethanesulphonic acid)], and Histopaque 1077 were purchased from Sigma (St Louis, MO, USA). 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) was from Molecular Probes (Eugene, OR, USA). Caspase-3 inhibitor zDEVD-fmk and caspase-8 inhibitor zIETD-fmk were from Calbiochem (Nottingham, UK), Dextran T500 was from Pharmacia (Uppsala, Sweden), and fetal bovine serum and Trizol reagent were from Gibco BRL (Gaithersburg, MD, USA).

Culture of *Trichomonas vaginalis*

The *Trichomonas vaginalis* isolate KT-4, used in the present study, was isolated from a Korean female with acute vaginitis (20). Parasites were grown in a complex trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (21).

Isolation of human neutrophils

Fresh human blood was drawn from healthy donors and treated with heparin, and neutrophils were isolated from it using a method previously described, with minor modifications (22). Briefly, 10 volumes of blood were mixed with 2 volumes of dextran [4·5% dextran T500 suspended in PIPES buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5·4 mM glucose, pH 7·4)]. After 30 min sedimentation at 37°C, neutrophils were obtained by layering on Ficoll-Hypaque (Histopaque 1077). After centrifuging at 385 *g* for 30 min at 4°C, the supernatant and mononuclear cells at the interface were carefully removed. The inside wall of the centrifuge tube was wiped twice with sterile gauze to remove adhering mononuclear cells. Erythrocytes in the sediment were lysed twice with sterile distilled water. Cell viability was determined using the trypan blue exclusion test (> 99%). Purity of neutrophils was confirmed morphologically (> 98%) and monocyte contamination was examined by phenotypic analysis using flow cytometry (Becton Dickinson, San Jose, CA, USA) after staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 Ab (< 0·02%).

Culture conditions for neutrophil apoptosis

Freshly isolated neutrophils were cultured in suspension with RPMI-1640 medium supplemented with 10 mM HEPES and 10% fetal bovine serum. In a preliminary experiment to obtain an optimal neutrophil/trichomonad ratio for enhanced apoptosis, neutrophils (1×10^6) were co-cultured with live trophozoites at various neutrophil/trichomonad ratios (50 : 1–10 : 1) for 12 h, and the optimal ratio for increased apoptosis was found to be 10 : 1; therefore 1×10^6 neutrophils were co-cultured with 1×10^5 live trophozoites in the subsequent experiments. For a positive control, neutrophils were treated with cycloheximide (20 µg/mL) to induce apoptosis.

To confirm whether close attachment of *T. vaginalis* to neutrophils was critical for induction of apoptosis, we used the 24-well Transwell insert system (Costar, Cambridge, MA, USA). These inserts, which have a porous (pore diameter 3 µm) membrane, serve as the upper chambers and ordinary tissue culture plate wells serve as the lower chambers. Medium containing trophozoites (2×10^5) was added to the upper chambers and neutrophil suspension (2×10^6) was added to the lower chamber. The plates were then incubated for 12 h, and apoptosis of neutrophils was determined.

To investigate whether adhesin protein of *T. vaginalis* affects neutrophil apoptosis, mAb to AP65 (a major adhesion protein of *T. vaginalis*) or a matched isotype control Ab (mouse IgG1, Sigma) was incubated with *T. vaginalis* at 37°C for 2 h. The mAb to AP65 was a mixture of 3 mAbs: anti-12G4 mAb, anti-DM116 mAb and anti-C55 mAb (23).

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After pre-incubation with the mAbs, the trichomonads were gently washed with culture medium prior to the 12 h co-culture with neutrophils.

Assays for neutrophil apoptosis

Morphologic assessment

Cellular morphology was ascertained by light microscopy following Giemsa staining of cytocentrifuged neutrophils. In addition, transmission electron microscopic observation was also carried out. A cytocentrifuge sample of 1×10^5 cells was prepared using a Shandon cytospin centrifuge (Shandon, UK) and was stained with Giemsa (12). Apoptosis was characterized by condensed and fragmented nuclei.

Transmission electron microscopy was conducted as follows: neutrophils (1×10^7) were incubated for 12 h, fixed with 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7·3) for 3 h, washed three times, and postfixed with 1% osmium tetroxide in 0·1 M phosphate buffer for 2 h. The specimens were then dehydrated in an ethanol gradient, embedded in Epon, and polymerized at 60°C for 48 h. Ultrathin sections (50–70 nm) were stained with 1% uranyl acetate followed by lead citrate, and examined under a transmission electron microscope (H-7600S, Hitachi, Japan) (24).

Flow cytometric analysis

FITC-conjugated annexin V, which binds to phosphatidylserine, and propidium iodide (PI) were added to 1×10^5 cell suspension according to the manufacturer's instructions (Apoptosis Detection Kit, R&D Systems, Minneapolis, MN, USA) and then incubated for 15 min at room temperature in the dark. Subsequently, the cells were analysed by flow cytometry. Early apoptotic cells were stained with annexin V alone, whereas necrotic cells and late apoptotic cells were stained with both annexin V and PI.

Reduced mitochondrial transmembrane potential is known to occur late in the apoptotic process. In the present study, mitochondrial transmembrane potential was assessed using $DiOC_6$ staining and flow cytometry. Briefly, neutrophils (2×10^6) were co-cultured with live trophozoites (2×10^5/mL) for 1–24 h and washed with cold PBS, after which cells (5×10^5/mL) were incubated with $DiOC_6$ (40 nM in PBS) for 15 min at 37°C (23). A total of 10 000 cells were analysed by a flow cytometry using a FACSCalibur with CELLQUEST PRO software (BD Bioscience, Germany). To distinguish neutrophils from *T. vaginalis*, forward and side scatter were simultaneously measured at each incubation time.

Determination of caspase-3 activity

Activation of caspase-3 was determined by detection of the chromophore p-nitroanilide (pNA) formed by the cleavage of the labelled caspase-3 substrate Asp-Glu-Val-Asp-pNA (DEVD-pNA) (ApoAlert caspase colorimetric assay kits, Clontech, CA, USA). In brief, 2×10^6 cells were lysed and incubated with DEVD-pNA for 1 h at 37°C. Optical density was measured at 405 nm, and activity of caspase-3 was estimated by the rate of pNA release (pM/min/2×10^6 cells). Control reactions in each experiment included the caspase-3 inhibitor zDEVD-fmk added prior to the addition of DEVD-pNA. This inhibitor completely blocks caspase-3 activation (25).

Western blot analysis of Bcl-2 family proteins

After neutrophils (2×10^6) were co-cultured with live *T. vaginalis* (2×10^5) for 5 h, 10 h and 20 h, cell lysates were prepared in ice-cold lysis buffer [20 mM Tris-HCl (pH 7·5) 200 µL, 60 mM β-glycerophosphate 600 µL, 10 mM EDTA 200 µL, 10 mM $MgCl_2$ 444 µL, 10 mM NaF 200 µL, 2 mM DTT 20 µL, 1 mM Na_3VO_4 50 µL, 1 mM APMSF 100 µL, 1% NP-40 100 µL, leupeptin (5 µg/mL) 50 µL, distilled water 8·036 mL, total volume 10 mL]. Twenty µg of each sample was electrophoresed in 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham, Sweden). After blocking overnight at 4°C with 5% skimmed milk, blots were incubated with primary antibodies [anti-Bax mAb, mouse anti-Bak polyclonal Ab (BD PharMingen, San Diego, CA, USA; 2 µg/mL), anti-Bcl-2 mAb, anti-Mcl-1 mAb (Oncogene, CA, USA; No. AM50, 1 : 200), and anti-actin mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA; No. sc-8432, 1 : 200) in 1% BSA Tris-buffered saline and 0·1% Tween–20] for 2 h at room temperature. Blots were then incubated with horseradish peroxidase-conjugated IgG (Amersham) for 1 h at room temperature and developed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Uppsala, Sweden) (25).

The relative quantity of each protein was determined by densitometric assay. The bands were analysed by QUANTITY ONE software (version 4·5·0; Bio-Rad, Hercules, CA, USA) and protein quantity was compared after density compensation with an actin control.

Statistical analysis

The results are expressed as mean ± SEM of three to five independent experiments. The Mann–Whitney *U*-test was used for statistical analysis, and a *P*-value of < 0.05 was considered statistically significant.

RESULTS

Trichomonas vaginalis-induced apoptosis in neutrophils

To determine whether *T. vaginalis* induces apoptosis of human neutrophils, neutrophils were incubated with *T. vaginalis* for

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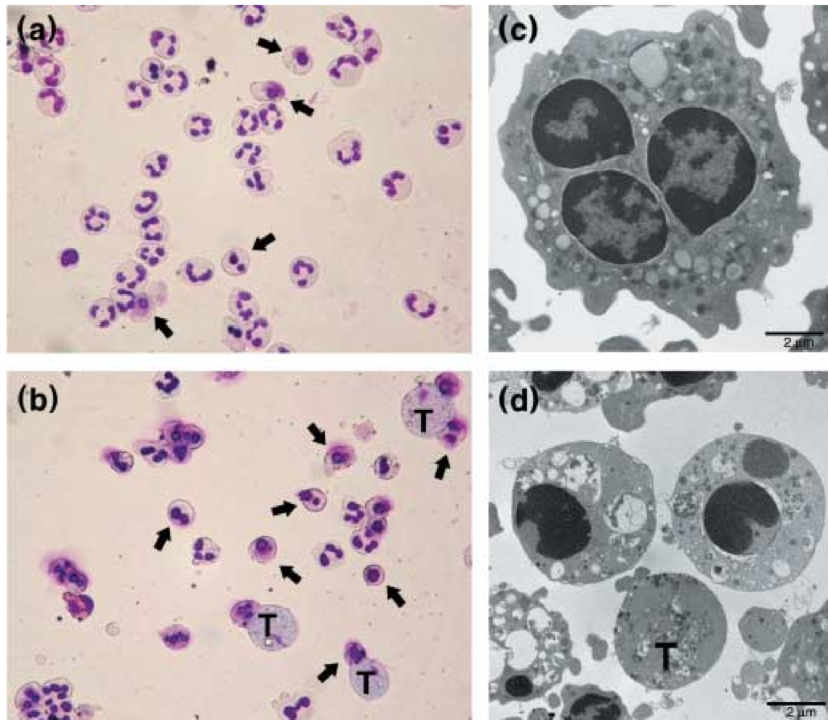

Figure 1 Trichomonas vaginalis induces apoptosis of human neutrophils. (a and b) Cytospin preparations of neutrophils were stained with Giemsa, and apoptotic cells (arrow) were seen morphologically (×400); (c and d) Transmission electron micrographs of neutrophils. The micrographs show neutrophils treated as follows: (a) neutrophils cultured with medium only for 12 h, (b) neutrophils co-cultured with live *Trichomonas vaginalis* (T) for 12 h, (c) freshly isolated normal neutrophils, (d) neutrophils and *T. vaginalis* were co-incubated for 12 h at 37°C. Several activated neutrophils surrounded one trichomonad (T). Two neutrophils show typical features of apoptosis (chromatin condensation).

12 h and their morphologies were examined using Giemsa staining. As shown in Figure 1(a), uninfected neutrophils underwent spontaneous apoptosis after 12 h, showing typical apoptotic morphological changes such as chromatin condensation, accompanied with loss of multilobular nuclear structure. However, when incubated with *T. vaginalis*, the apoptotic process was markedly accelerated (Figure 1b). Transmission electron microscopy showed the fine ultrastructural apoptotic morphologies of neutrophils, including chromatin condensation, crescent-shape chromatin margination, the coalescence of nuclear lobes into a single body, and preservation of cytoplasmic granules and cell membrane integrity (Figure 1c,d).

The morphological assessments of apoptosis were further confirmed by two flow cytometric methods. One was flow cytometry using FITC-annexin V stained cells to detect the externalization of phosphatidylserine to the outer leaflet of the cell membrane, and the other was flow cytometry after $DiOC_6$ staining to detect reduced mitochondrial transmembrane potentials (Figure 2b). Neutrophils were distinguished from *T. vaginalis* by cell size and granularity in flow cytometric analysis (open circle; Figure 2a). The two staining methods (annexin V and $DiOC_6$) showed similar levels of apoptosis. $DiOC_6$ staining revealed that the spontaneous apoptosis of neutrophils without *T. vaginalis* increased with time (8·7% at 9 h and 19·6% at 12 h). When treated with *T. vaginalis*, apoptosis of neutrophils was accelerated showing significant increases of apoptosis at 12 h (26·1% at 9 h and 42·0% at 12 h) (Figure 3). Based on these results, subsequent experiments were performed by treating neutrophils with *T. vaginalis* for 12 h.

The level of neutrophil apoptosis caused by live *T. vaginalis* was found to be dependent on the number of live trichomonads used. When 1×10^6 neutrophils were incubated with 2×10^4, 5×10^4, or 1×10^5 trichomonads (in neutrophil/trichomonads ratios of 50 : 1, 20 : 1, or 10 : 1) for up to 12 h, the level of apoptosis was enhanced as the number of trophozoites increased (data not shown).

We reported previously that adherence between *T. vaginalis* and neutrophils is required for stimulation of IL-8 production by neutrophils (10). Adhesin proteins of *T. vaginalis* are known to be one out of multiple factors involved in adherence of *T. vaginalis* to vaginal epithelial cells (26–30). As shown in Figure 4, when *T. vaginalis* was pre-treated with an Ab against adhesin (anti-AP65 mAb), the apoptosis of neutrophils was reduced compared with neutrophils incubated with untreated trichomonads. Neutrophil apoptosis induced by trichomonads pre-treated with a matched isotype control Ab was similar to that induced by *T. vaginalis* without treatment.

Next, the question of whether contact between *T. vaginalis* and neutrophils is critical for apoptotic induction was examined using the Transwell insert system. When suspensions containing trophozoites and neutrophils were placed in the upper and lower wells, respectively, and incubated for 12 h,

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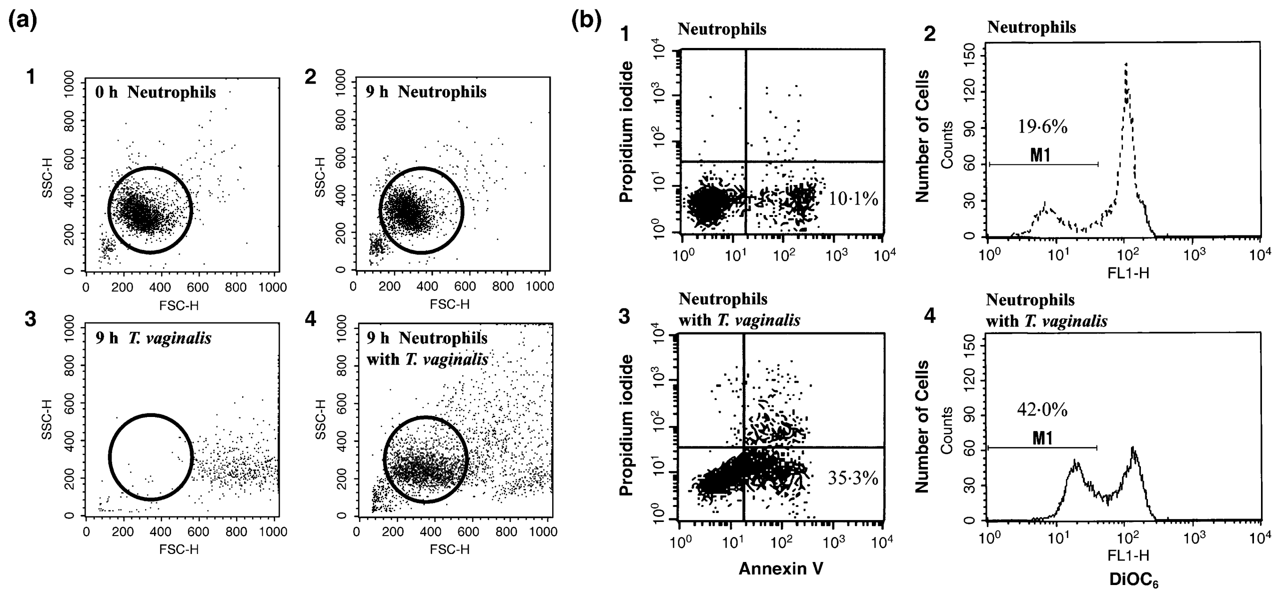

Figure 2 Flow cytometric analysis of neutrophils co-incubated with live *Trichomonas vaginalis*. (a) Identification of neutrophils and *T. vaginalis* by forward/sideward scatter signals. Panels are as follows: (1) freshly isolated neutrophils, (2) neutrophils and (3) trichomonads cultured with medium only for 9 h, (4) neutrophils co-cultured with live trichomonads for 9 h. Open circles represent neutrophils selected for flow cytometric analysis. (b) Neutrophils were incubated with fluorescein isothiocyanate-conjugated annexin V, propidium iodide (1 and 3), and 40 nM $DiOC_6$ (2 and 4) and analysed by flow cytometry. The area marked M1 contains the apoptotic neutrophil population. Neutrophils cultured with medium only for 12 h are shown in 1 and 2. Neutrophils co-cultured with *T. vaginalis* for 12 h are shown in 3 and 4.

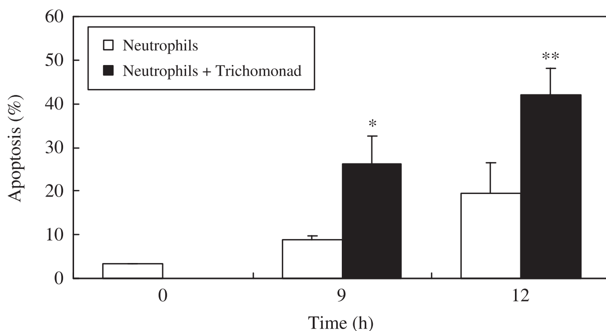

Figure 3 Time course of apoptosis of human neutrophils co-incubated with *Trichomonas vaginalis*. Neutrophils (1×10^6/mL) were co-cultured with live *T. vaginalis* (1×10^5/mL) for 0–12 h. The percentage of apoptotic cells was obtained by flow cytometry using $DiOC_6$. The data represent mean ± SEM of four separate experiments. **P* < 0·05; ***P* < 0·01, Neutrophils vs. Neutrophils + *T. vaginalis*.

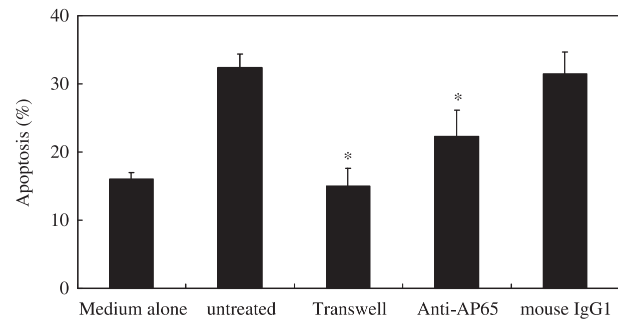

Figure 4 Effect of the adherence of *Trichomonas vaginalis* to neutrophils on the induction of neutrophil apoptosis. The Transwell chamber was used to prevent adhesion; trophozoites and neutrophils were placed in the upper and lower well, respectively, and incubated for 12 h. 'Medium alone' represents neutrophils incubated without *T. vaginalis*; 'untreated' represents neutrophils plus *T. vaginalis* in the absence of Ab. Apoptosis was also assessed for neutrophils incubated with *T. vaginalis* pretreated with mAb to adhesin protein AP65 or with an isotype-matched control Ab (mouse IgG1). The percentage of apoptotic cells was determined by flow cytometry using $DiOC_6$. The data represent mean ± SEM of four separate experiments. **P* < 0·05; control vs. treatment.

the level of neutrophil apoptosis in the lower well was almost the same as that for neutrophils cultured without parasites (medium alone) (Figure 4).

Activation of caspase-3 in *T. vaginalis*-induced apoptosis

Caspase-3 is a key enzyme in the spontaneous apoptosis of neutrophils (31). The enzymatic activity of caspase-3 in lysates of neutrophils was measured using a synthetic substrate of caspase-3 (DEVD-pNA), which gives rise to a fluorescent product after cleavage by the activated enzyme. The caspase-3 activity of neutrophils alone incubated for 6 h and

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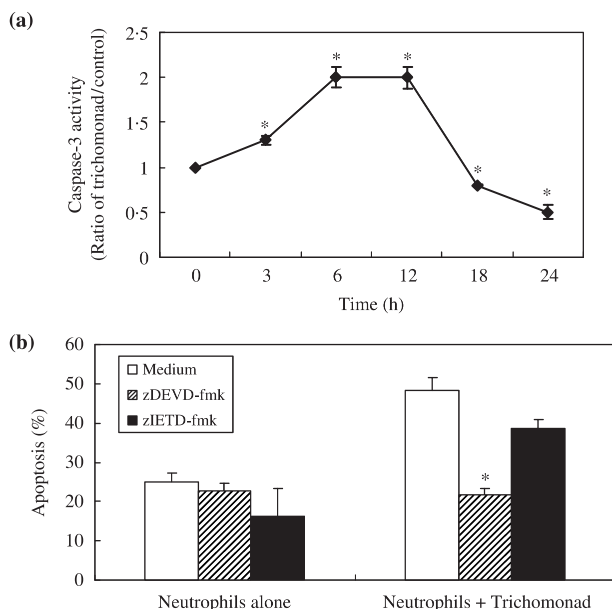

Figure 5 Analysis of caspase-3 activity in neutrophils and effect of caspase inhibitors. (a) Activation of caspase-3 was determined by detection of chromophore p-nitroanilide (pNA) after cleavage of the labelled substrate DEVD-pNA. After co-cultivation of neutrophils with *Trichomonas vaginalis* for 24 h, cell lysate supernatants were prepared at the designated times. The supernatants were incubated with DEVD-pNA for 1 h at 37°C. Optical density was measured at 405 nm and presented as the ratio of *T. vaginalis*-treated neutrophils/untreated neutrophils. $^*P < 0{\cdot}05$; significant difference from control group. (b) Effect of inhibitors of caspase-3 and caspase-8 on spontaneous and *T. vaginalis*-induced neutrophil apoptosis. Neutrophils (1×10^6/mL) were pre-treated for 10 min at 37°C with 25 mM zDEVD-fmk or zIETD-fmk to inhibit caspase-3 and 8, respectively. The cells were subsequently incubated with *T. vaginalis* (neutrophils/*T. vaginalis*, 10 : 1) or in medium alone and then cultured for 12 h. Untreated cells (no inhibitors) were cultured with or without trichomonads for the same period of time. The percentage of apoptotic cells was obtained by flow cytometry using $DiOC_6$. The data represent mean ± SEM of four separate experiments. (*, significantly different from cells not exposed to inhibitors; $P < 0{\cdot}05$)

12 h was 27·4 and 48·4 (pM/min/2×10^6 cells), respectively. *Trichomonas vaginalis* treatment doubled the level of caspase-3 activity compared to untreated neutrophils at 6 h and 12 h (54·8 and 95·9 pM/min/2×10^6 cells, respectively). At 18 h and 24 h, caspase-3 activity had decreased from the level at 12 h (Figure 5a).

We then attempted to identify the caspases involved in *T. vaginalis*-induced apoptosis in neutrophils by using different caspase inhibitors. Thus, neutrophils were pre-treated with 25 μM zDEVD-fmk or zIETD-fmk, which inhibit caspase-3 and -8, respectively (25), and then the cells were exposed to *T. vaginalis* for 12 h. As shown in Figure 5(b), the inhibition of caspase-3 activity effectively reduced *T. vaginalis*-induced apoptosis from 48·2% (neutrophils alone) to 21·7% (neutrophils and zDEVD-fmk). Spontaneous apoptosis was also partially inhibited by zDEVD-fmk. In contrast, *T. vaginalis*-induced apoptosis was only slightly reduced by the caspase-8 inhibitor, zIETD-fmk, indicating the major involvement of caspase-3 in the neutrophil apoptosis.

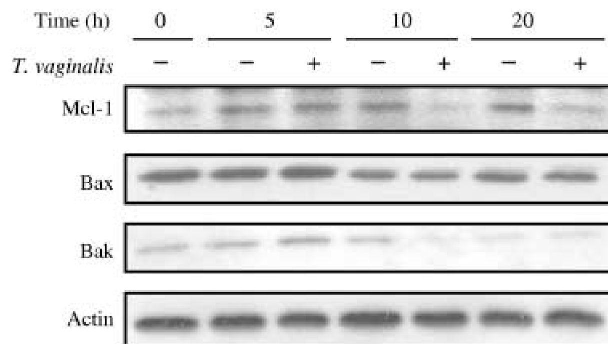

Figure 6 Western blot analysis of Bcl-2 family protein expression. After neutrophils were co-cultured with *T. vaginalis* for 5, 10 and 20 h, cell lysates were electrophoresed in 12% SDS-PAGE and electroblotted on nitrocellulose membranes. Blots were incubated with primary and horseradish peroxidase-conjugated secondary antibodies and then developed using an enhanced chemiluminescence detection kit. Representative Western blots of three separate experiments are shown.

Mcl-1 attenuation in neutrophils activated by *T. vaginalis*

To determine the changes of Bcl-2 family proteins, Western blotting was performed on protein extracts of neutrophils cultured in media alone or activated with *T. vaginalis*. An anti-apoptotic protein, Mcl-1 (13), expression was found to decrease in neutrophils incubated with *T. vaginalis* for 10 h and 20 h. The ratios of relative protein intensity (neutrophils with *T. vaginalis*/neutrophils only) were 1·10, 0·54 and 0·63 at 5 h, 10 h, and 20 h, respectively. By contrast, Bax and Bak protein levels were not different between *T. vaginalis*-treated and untreated neutrophils, although decreased intensity of bands was observed in all cell preparations after 10 h (Figure 6). Bcl-2 expression was undetectable in both infected and uninfected neutrophils.

DISCUSSION

In the present study employing light and electron microscopy and flow cytometry, live *T. vaginalis* was found to increase neutrophil apoptosis. Flow cytometric analysis using both annexin V-PI and $DiOC_6$ staining showed similar rates of neutrophil apoptosis by both methods. Annexin V binds phosphatidylserine, which is exposed in the outer leaflet of the phospholipid bilayer during apoptosis or necrosis. Cells

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that retain membrane integrity, including viable and early apoptotic cells, do not take up PI. Therefore, the combined use of annexin and PI can be used to distinguish early-apoptotic from late-apoptotic or necrotic cells. $DiOC_6$ staining was employed to follow changes of mitochondrial transmembrane potential in apoptotic neutrophils. Flow cytometric measurement of neutrophil apoptosis induced by interaction between neutrophil and live *T. vaginalis* has been thought to be difficult due to the similar sizes of *T. vaginalis* and neutrophils making it difficult to identify the two cell populations. However, we show that trichomonads can be differentiated from neutrophils on the basis of their higher forward scatter allowing each of the two populations to be gated separately.

An mAb against AP65, one of the four major adhesin proteins of *T. vaginalis*, reduced the level of neutrophil apoptosis induced by the parasite. The apoptosis-delaying effect of this anti-adhesin mAb is probably due to the inhibition of trichomonad adherence to neutrophils, since anti-AP65 mAb has been reported to reduce *T. vaginalis* adherence to vaginal epithelial cells (26). Using a Transwell chamber, we investigated the importance of contact between neutrophils and *T. vaginalis* for neutrophil apoptosis. The results of the Transwell experiments indicate that direct contact between neutrophils and *T. vaginalis* – most likely mediated by AP65 – is essential for neutrophil apoptosis to occur.

Human neutrophils are known to express the mRNAs of anti-apoptotic proteins, such as Mcl-1 and A1 (13), and levels of Mcl-1 protein have been reported to correlate with neutrophil survival (32,33). When neutrophils were pre-treated with GM-CSF or LPS (both of which are known to delay neutrophil apoptosis), Mcl-1 protein expression was increased (13). In our study, Mcl-1 protein levels in neutrophils were decreased after incubation with *T. vaginalis*. This result is in agreement with the finding of Moulding *et al.* (32) in which levels of Mcl-1 protein decreased prior to the onset of apoptosis.

The execution of the apoptotic pathway is mediated by caspases and human neutrophils express several different caspases; however, caspase-3 and -8 are known to be mainly activated during apoptosis (11). We found that the inhibition of caspase-3, but not caspase-8, effectively blocked *T. vaginalis*-induced apoptosis of neutrophils, and that stimulation with trichomonads markedly up-regulated caspase-3 activity. This suggests that *T. vaginalis*-induced apoptosis of neutrophil is mediated primarily through caspase-3 activation.

There are several studies that examine the role of increased neutrophil apoptosis in the pathogenesis of a variety of infectious diseases (34–37). Induction of apoptosis of cells associated with innate or adaptive immunity would appear to offer a means of countering a host antimicrobial defence measure. For example, induction of the apoptotic death of mononuclear and polymorphonuclear leucocytes by *Fusobacterium nucleatum* has been suggested to mediate immunosuppression in periodontal disease (38). It is also known that the apoptosis of neutrophils following phagocytosis by macrophages or fibroblasts provides a means of safely removing neutrophils from sites of inflammation, thus minimizing the risk of bystander tissue damage. This safe removal of neutrophils is essentially required for resolution of inflammation (39,40). Neutrophil infiltration is a ubiquitous feature of *T. vaginalis*-associated vaginitis (41,42) and is considered to be primarily responsible for the cytological damage (1,43); however, its role in the pathogenesis of trichomoniasis has not yet been clearly characterized.

In our previous study, we observed the production of IL-8 and Gro-α by neutrophils when stimulated with live *T. vaginalis* (10); these cytokines may subsequently induce more infiltration and recruitment of neutrophils by chemotaxis at the reaction site, and neutrophil accumulations are thought to cause continued inflammation and/or aggravated vaginal inflammation. In the present study, *T. vaginalis* was found to increase neutrophil apoptosis, which may contribute to the resolution of acute trichomoniasis. We are in the process of determining whether macrophages can phagocytose the apoptotic neutrophils induced by *T. vaginalis*. This would strongly suggest that increased neutrophil apoptosis by *T. vaginalis* contributes to the resolution of inflammation.

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