MYCOLOGY

Early membrane exposure of phosphatidylserine followed by late necrosis in murine macrophages induced by Candida albicans from an HIV-infected individual

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The hypothesis that Candida albicans isolate (CR1) from an HIV-infected individual induced apoptosis of macrophages was examined by optical microscopy, binding of annexin V-FITC and analyses of DNA degradation (TUNEL tests and agarose gel electrophoresis). Resident murine peritoneal macrophages co-incubated for 5–15 min with C. albicans CR1 bound annexin V, whereas macrophages incubated with either heat-inactivated strain CR1, C. albicans 577 (isolated from a patient with mucocutaneous candidiasis) or C. albicans FCF14 (a mutant that did not produce proteases and phospholipases) did not bind annexin for up to 2 h of observation. However, macrophages exposed to C. albicans CR1 did not present the pattern of DNA degradation typical of apoptosis. Macrophages became increasingly permeable to propidium iodide from 30 min to 2 h after their exposure to C. albicans CR1. Most of the phagocytosed C. albicans CR1 yeast cells switched to germ-tubes inside the macrophages after incubation for 1–2 h. These results show that macrophages exposed to C. albicans CR1 presented early signs of apoptosis but progressed to necrosis, and suggest that Candida strains that readily switch to germ-tubes inside those apoptotic cells might have a competitive advantage in vivo because released germ-tubes resist further attack by macrophages.

Introduction

Following the first demonstration that Shigella flexneri was able to induce macrophage apoptosis in vitro [1], several other bacteria were also shown to induce apoptosis [2–7]. It was usually observed that the bacteria must be phagocytosed and viable to induce apoptosis, although some bacterial proteins and an HIV protein induced apoptosis by themselves [8–11]. Induction of apoptosis by bacterial infection in vivo was also demonstrated and the cell death and ensuing changes in the secretion of cytokines may be important in microbial pathogenesis [12, 13]. These observations led to the concept that the ability of pathogens to induce apoptosis of phagocytes might be an important virulence factor, for it would curtail the host’s defence mechanisms.

Although innate cellular immunity is of prime importance for the host defence against fungal infections, it is not known whether Candida albicans and other fungi can exploit phagocyte apoptosis to their own advantage. Phagocytosis of heat-killed C. albicans increased the apoptosis of human neutrophils after culture for 18 h [14]. In contrast, infection by C. albicans inhibited apoptosis of human monocytes and supernates from co-cultures of neutrophils and C. albicans inhibited apoptosis of naive neutrophils [14–16].

Previous studies showed that murine peritoneal macrophages phagocytose C. albicans in vitro through both complement and mannose receptors and that concanavalin A-activated macrophages have an increased ability to ingest and kill C. albicans [17–19]. In preliminary experiments aimed at characterising the ability of macrophages to phagocytose and kill a seemingly more virulent strain of C. albicans recently isolated from an HIV-infected individual, this strain caused complete destruction of macrophage mono-
layers after co-incubation for 2 h; however, when observed after co-incubation for only 30 min these macrophages had phagocytosed several yeast cells and some of their nuclei showed peripheral condensations resembling those seen at the beginning of apoptosis. Therefore, the possibility that these macrophages might have entered into apoptosis and were subsequently lysed was investigated in the present study.

Materials and methods

Micro-organisms and cultivation

*C. albicans* 577, isolated from the skin of a patient with mucocutaneous candidiasis, *C. albicans* CR1, isolated from the oral mucosa of an HIV-infected patient at the Dentistry School, Universidade Estadual de Londrina, Brazil, and *C. albicans* FCF14, a mutant that did not produce proteases and phospholipases (kindly provided by Dr M. T. Shimizu, School of Dentistry, Universidade Estadual Paulista, Botucatu, Brazil) were used in this study. The fungi were maintained on Sabouraud dextrose agar; fungal cells were obtained by growth in Sabouraud dextrose broth (Difco) for 24 h at 28 °C, harvested by centrifugation (2000 g, 6 min), washed three times with phosphate-buffered saline (PBS) and resuspended at 4 × 10⁶ cells/ml in RPMI 1640 medium. The blastoconidia were resuspended at 4°C with agitation (120 rpm). The blastoconidia were harvested by centrifugation (2000 g, 6 min), washed three times with phosphate-buffered saline (PBS) and resuspended at 4 × 10⁶ cells/ml in RPMI 1640 medium (Sigma) containing bovine serum albumin 1%.

Heat-inactivated *C. albicans* were used in some experiments and were obtained by incubating 4 × 10⁶ cells/ml in PBS at 60°C with agitation (120 rpm). The blastoconidia were harvested by centrifugation (2000 g, 6 min), washed three times with phosphate-buffered saline (PBS) and resuspended at 4 × 10⁶ cells/ml in RPMI 1640 medium (Sigma) containing bovine serum albumin 1%.

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C. albicans were opsonised immediately before the phagocytosis experiments by incubating 4 × 10⁶ cells in 1 ml of RPMI medium containing fresh non-immune mouse serum 2.5% for 5 min at 37°C; these cells were then cytospun and resuspended in RPMI medium.

Avian *Escherichia coli* UEL 17 and *Shigella flexneri* M90T (kindly supplied by P. I. Sansonetti) were maintained on Luria-Bertani agar and were grown to 10⁸ bacteria/ml in RPMI 1640 medium at 37°C.

Mouse peritoneal macrophages

Resident peritoneal phagocytes were collected by rinsing the peritoneal cavity of male Swiss mice (weighing c. 30 g) with 3 ml of RPMI 1640 medium containing BSA 1%. The cells were cytospun, resuspended in RPMI medium, counted in a haemocytometer, and the concentration was adjusted to 4 × 10⁶ cells/ml. The cells were allowed to adhere to glass covers for 1 h at 37°C under an atmosphere of O₂ 95%, CO₂ 5% and the preparation was then rinsed with RPMI 1640 medium to remove non-adherent cells. The cell monolayer adhered to the glass cover contained almost exclusively macrophages.

Apoptosis assays

Cell monolayers were co-incubated with *C. albicans* (10 yeast cells/macrohage) for 5 or 15 min in RPMI medium at 37°C, rinsed three times with the same medium and then either immediately processed for detection of apoptosis or re-incubated for up to 2 h before testing for apoptosis. The following tests were applied to the cell monolayers for detection of apoptosis: (1) optical microscopy analysis after staining with May-Grumwald-Giemsa; (2) fluorescence microscopy analysis of surface exposure of phosphatidylserine by means of the TACS annexin V-FITC kit (R&D Systems, Minneapolis, MN, USA); and (3) in-situ terminal deoxynucleotidyl transferase nick end-labeling assay (TUNEL) by means of the TACS TdT kit (R&D Systems). Either *S. flexneri* M90T or *E. coli* UEL 17 (10⁸ bacteria/ml) were used as positive controls of apoptosis induction.

For analysis of DNA fragmentation, macrophages (10⁶ cells/ml, in RPMI 1640 medium) were allowed to adhere to round-bottomed wells (Costar plates) for 1 h at 37°C, co-incubated for 15 min with 10⁷ *C. albicans* cells, and then rinsed and re-incubated for up to 4 h. DNA of these cells was extracted by the phenol-chloroform method [20], fractionated in agarose 1% gel in Tris-borate-EDTA buffer (c. 7–12 μg of DNA per lane), stained with ethidium bromide and photographed on an Image Master (Pharmacia).

Staining by propidium iodide

Macrophages co-incubated with *C. albicans* for 15 min, according to a protocol similar to the one described above, were re-incubated for up to 2 h and then stained with propidium iodide (TACS annexin V-FITC kit).

Results

Morphology of macrophages and formation of germ-tubes by *C. albicans* CR1

Macrophages displayed several phagocytosed yeast cells contained within clearly delimited vesicles 15–30 min (Fig. 1a) after their initial exposure to *C. albicans* CR1, and many of them had distorted nuclei and peripheral nuclear condensation. After 60 min, *C. albicans* CR1 had frequently formed germ-tubes inside macrophages (Fig. 1b); after 120 min abundant hyphae had formed and the number of cells that remained attached to the glass slides was reduced. Macrophages co-incubated with *C. albicans* 577 rarely had germ-tubes in their interior after 60 min (Fig. 1c) and after 120 min no hyphae had formed around them. Both heat-inactivated *C. albicans* CR1 and viable *C. albicans* FCF14 were phagocytosed, but neither caused...
evident peripheral condensations in the nuclei of the cells that phagocytosed them, for up to 2 h of observation, and the latter strain did not form germ-tubes inside macrophages.

Binding of annexin V-FITC to macrophages

Several macrophages in each field bound annexin V-FITC after co-incubation with C. albicans CR1 for 5 min (Fig. 2a), although many macrophages had not yet phagocytosed any yeast cells after that short period of incubation. After 15 min, many macrophages co-incubated with C. albicans CR1 were labelled by annexin V-FITC (Fig. 2b), whereas macrophages co-incubated with C. albicans 577 were not labelled after that period (Fig. 2c) or after further incubation for up to 2 h. Macrophages that phagocytosed heat-inactivated C. albicans CR1 did not bind annexin V-FITC, although several macrophages contained a large number of ingested yeast cells after incubation for 15 min. Macrophages co-incubated with C. albicans FCF-14 for periods of up to 2 h were not significantly labelled by annexin V-FITC.

Macrophage labelling by propidium iodide

Macrophages incubated with C. albicans CR1 became progressively permeable to propidium iodide. After exposure to C. albicans CR1 for 15 min, few macrophages were labelled by propidium iodide, but after 60 min almost all macrophages in each field were labelled by both propidium iodide and annexin V-FITC (Fig. 3a); yeast cells unstained by propidium iodide could be seen inside macrophages. This increase in macrophage permeability seemed to occur mostly from 30 min (Fig. 3b) to 60 min (Fig. 3c) after exposure to C. albicans CR1, as shown by cells labelled only with propidium iodide. Co-incubation of macrophages with C. albicans CR1...
577 did not cause a similar increase in permeability after 60 min. Macrophages were swollen (Fig. 3d) and entangled in the growing hyphae (Fig. 3e) 120 min after their initial exposure to

\textit{C. albicans} CR1. Co-incubation of macrophages with either heat-inactivated \textit{C. albicans} CR1 or \textit{C. albicans} FCF14 did not cause an increase in their permeability to propidium iodide similar to that caused by co-incubation with \textit{C. albicans} CR1.

\section*{Effect of pepstatin-A on macrophage labelling by annexin V-FITC and propidium iodide}

Macrophages co-incubated with pepstatin-A for 20 min were not labelled by annexin V-FITC after co-incubation with \textit{C. albicans} CR1 for 15 min, and were rarely labelled after 30 min. These macrophages became permeable to propidium iodide from 30 to 60 min after initial exposure to \textit{C. albicans} CR1.

\section*{TUNEL assays and DNA fragmentation}

Macrophages co-incubated with \textit{C. albicans} CR1 did not bind the streptavidin-FITC conjugate in TUNEL assays performed 60 or 120 min (Fig. 4a) after their initial exposure to the yeast; TUNEL assays performed at 5, 15 or 30 min were also negative. Macrophages treated with TACS-nuclease (positive control), in each experiment, were labelled after incubation for 60 min (Fig. 4b) or 120 min, and macrophages infected with \textit{S. flexneri} were labelled after incubation for 4 h. Agarose gel electrophoresis of DNA extracted from macrophages incubated with \textit{C. albicans} CR1 did not show the ladder pattern of DNA degradation characteristic of apoptosis at any of the incubation periods tested (5, 15, 30, 60, 120 and 240 min), whereas DNA from the positive control obtained by co-incubation with \textit{S. flexneri} showed the apoptotic pattern.

\section*{Discussion}

Several studies have shown that strains of \textit{C. albicans} isolated from HIV-infected individuals had increased expression of virulence traits such as proteinase production, adherence, resistance to antifungal drugs and phenotypic variation [21–23]; these observations support the conclusion that more virulent strains are either selected from commensal strains or replace them concomitantly with the lowering of the host’s cellular defences. In this study, a strain of \textit{C. albicans} (CR1)
APOPTOSIS AND NECROSIS INDUCED BY C. ALBICANS

Fig. 3. (Continued on next page)
Fig. 3. Fluorescence micrographs of macrophages labelled with propidium iodide and annexin V-FITC. (a) Macrophages co-incubated with C. albicans CR1 for 15 min rinsed and re-incubated to complete 60 min and labelled with propidium iodide and annexin V-FITC; (b) macrophages co-incubated with C. albicans CR1 for 15 min, rinsed and re-incubated to complete 30 min or (c) 60 min, and labelled with propidium iodide. (d) Macrophages labelled with propidium iodide 120 min after their initial exposure to C. albicans CR1, and (e) brightfield image from the same field as (d). Representative results of at least three independent experiments. Magnification: a, b, 320×; c, 360×; d, e, 900×.

Fig. 4. (a) Fluorescence micrograph of TUNEL reactions applied to macrophages 120 min after their initial exposure to C. albicans CR1. Magnification 400×. (b) Macrophages treated with nuclease (positive control) and tested by TUNEL after incubation for 60 min. Representative results of at least five independent experiments. Magnification 370×.
isolated from an HIV-infected person induced mem-
brane exposure of phosphatidylserine by murine macrophages after co-incubation for only 5 to 15 min, which suggests that this strain exploits induction of apoptosis as a virulence factor. Although these macro-
phages had many phagocytosed yeast cells within clearly delimited vesicles 15–30 min after exposure to C. albicans CR1, after 1–2 h germ-tubes and hyphae developed and the macrophages were either permeable to propidium iodide or completely lysed. Neither heat-
inactivated C. albicans CR1 nor viable C. albicans 577 induced exposure of phosphatidylserine on the macro-
phage membranes, as judged from the absence of annexin V-FITC binding. Moreover, macrophages that phagocytosed heat-inactivated C. albicans CR1 re-
mained impermeable to propidium iodide for up to 2 h of observation and did not present signs of chromatin condensation. C. albicans 577 rarely formed germ-
tubes inside macrophages. Although these macrophage monolayers were better preserved, some cells were permeable to propidium iodide after incubation for 1–
2 h. These in-vitro observations support the inference that C. albicans CR1 uses induction of apoptosis and switching to germ-tubes inside macrophages as a mechanism to evade the host’s defences. However, to estimate whether this is a trait common in virulent strains, the ability of a large number of Candida strains to induce apoptosis and switch to germ-tubes inside host cells should be evaluated.

Secretion of aspartyl proteinases is considered to be an important virulence trait of C. albicans, and strain CR1 produces proteases and phospholipases, as detected on agar plates (unpublished results). Macrophages pre-
incubated with the proteinase inhibitor pepstatin A had
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Both exposure of phosphatidylserine and internucleo-
sonal degradation of DNA are hallmarks of apoptosis. However, repeated TUNEL tests conducted at several
time intervals after exposure of macrophages to C.
albicans CR1 did not show any significant labelling, indicating the absence of degraded DNA nick ends, although the positive controls always confirmed that the
reaction worked properly. Absence of the DNA
degradation typical of apoptosis was also repeatedly
confirmed by agarose gel electrophoresis. These results
might indicate that apoptosis is initially triggered and
then interrupted by further interactions between the
phagocytosed yeasts and the macrophage. Another
possible explanation is that C. albicans CR1 induces
apoptosis through the recently discovered caspase-
independent pathway of programmed cell death, in
which apoptosis-inducing factor (AIF) released from
the mitochondria acts directly on the cell nucleus and
leads to degradation of DNA into large fragments of c.
50 kb [24, 25]. As AIF causes nuclear chromatin
condensation, this last explanation also encompasses
the initial observation that macrophages showed
chromatin condensation 30 min after exposure to C.
albicans CR1.

Several studies have demonstrated induction of apo-
ptosis of macrophages and other cell lines a few hours
after bacterial infection in vitro [1–7], but in this study
exposure of phosphatidylserine by macrophages was
observed within a few minutes of their contact with C.
albicans CR1. However, the macrophages did not
progress into the well-known sequence of apoptotic
changes and, after 1–2 h almost all of them were either
necrotic or lysed. This suggests that apoptotic cells,
which have their mitochondrial functions impaired,
might provide an adequate temporary environment for
the switching of yeast cells to germ-tubes. If some
strains of Candida are able to induce apoptosis of
macrophages in vivo, yeast strains that readily switch
to germ-tubes inside those apoptotic cells would have a
competitive advantage, because germ-tubes released by
cell lysis are more resistant than yeast cells to further
attack by incoming macrophages.

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