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Membrane changes associated with the early stages of apoptosis in HEp-2 cells decrease susceptibility to adherence by *Candida albicans*

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The aim of the present work was to establish whether apoptotic HEp-2 cells demonstrated an altered susceptibility to adherence by the pathogenic yeast *Candida albicans*. Cultures of HEp-2 cells were treated with concentrations of three chemotherapeutic agents sufficient to induce cell death by apoptosis and this was confirmed by microscopic examination and by the loss of membrane asymmetry (as indicated by phosphatidylserine externalization) and the fragmentation of nuclear DNA into distinct subunits. Cells in the early stages of apoptosis demonstrated a reduced susceptibility to adherence by a number of strains of *C. albicans*. A correlation between the decrease in yeast adherence and the loss of membrane asymmetry, associated with the early stages of apoptosis, was established.

Keywords adherence, apoptosis, *Candida*, HEp-2

Introduction

The ability of *C. albicans* to adhere to a range of host tissues is critical to the success of the pathogenic process since adherence is a necessary prerequisite to colonisation and infection [1,2]. Adherence also facilitates the persistence of the yeast in environments which may be hostile to its presence [3]. Adherence to host tissue is accomplished by a combination of specific and non-specific mechanisms. The former are the dominant contributors to the process and include a range of ligand–receptor interactions [3,4]. Non-specific mechanisms include electrostatic interactions, cell surface hydrophobicity and aggregation [5,6]. While it is clear that these latter forces contribute to the process of yeast adherence, it is generally accepted that their overall contribution is less than that of the specific mechanisms [7].

The aim of the work presented in this paper was to establish whether HEp-2 cells in the early stages of apoptosis displayed altered susceptibility to yeast adherence. Apoptosis is a form of cell death biochemically and morphologically distinct from necrosis. It may occur as part of the normal developmental processes involved in tissue formation or as a result of a variety of cellular stimuli or insults [8]. Apoptosis may be the dominant mode of cell death in vertebrates and has the advantage, when compared with necrosis, of not inducing an immune response. This form of cell death is characterized by the fragmentation of nuclear DNA into distinct nucleosome sized subunits, due to the activation of an endogenous endonuclease, and membrane blebbing which results in the formation of apoptotic bodies [9]. These are recognized and engulfed by phagocytes without the release of intracellular components which would evoke an immune response [10].

One of the earliest stages in the apoptotic process is the loss of membrane asymmetry as a result of the down-regulation of the ATP-dependent amino-phospholipid translocase and activation of a non-specific lipid scramblase [11]. This process occurs without concomitant membrane rupture in lymphocytes [12] or adherent cell lines [13] and before cell shrinkage or chromatin condensation [14]. One consequence of the loss of membrane asymmetry is the appearance of phosphatidyl serine (PS) on the outer leaflet of the lipid bilayer [15]. Flow cytometric detection of the binding of FITC-Annexin V to PS has been employed to measure the increased PS levels in the outer leaflet of the cell membrane of cells undergoing apoptosis [12]. Externalization of PS during the early stages of apoptosis has been demonstrated in a range of human and murine cell types [14].

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In this study, apoptosis was induced in HEp-2 cells by exposure to a number of anticancer drugs and confirmed by the loss of membrane asymmetry and DNA cleavage. Three cancer chemotherapeutic agents were chosen for this work.

Doxorubicin is a widely used anticancer drug effective against a range of tumours. Its principal mode of action lies in the ability to interact with cellular DNA although other mechanisms, including interference with the action of DNA topoisomerase II, have been demonstrated [16]. Methotrexate acts as an inhibitor of DNA synthesis by blocking dihydrofolate reductase [17]. Cisplatin kills cells by covalently interacting with and crosslinking DNA. Cells treated with this drug proceed to G_2 and undergo aberrant mitosis which leads to unequal chromosome distribution [18]. The three anticancer drugs employed in this study have previously been shown to kill cells by inducing apoptosis [16–18].

Materials and methods

Yeast strain and culture conditions

Candida albicans ATCC 44990, ATCC 10231 and strain MEN (serotype B, originally isolated from an eye infection) were used in this study. Cultures were grown overnight to the stationary phase ($1–2 \times 10^8$/ml) in YEPD (2% (w:v) glucose, 2% (w:v) Bactopeptone and 1% (w:v) yeast extract) at 37 °C and 200 rev min^{-1} in an orbital incubator.

Cancer cell line

The HEp-2 cell line (ATCC CCL23, obtained from the American Type Culture Collection, MD), derived from an epidermoid carcinoma of the larynx [19], was cultured in MEM (Sigma) supplemented with 5% (v:v) fetal calf serum (Gibco), 4 mM L-glutamine (Gibco) and 1% (v:v) Penn-Strep (Sigma). The adherent cells were grown in 80 cm^2 tissue culture flasks (Nunc) at 37 °C and subcultured by trypsinization every 3–4 days.

Anticancer drugs

Doxorubicin and cisplatin were obtained from Farmitalia Carlo Erba (Milan, Italy). Methotrexate was purchased from Lederle (Hampshire, England).

Toxicity assay

Subconfluent HEp-2 cells were harvested by trypsinization, washed and resuspended in PBS. Cells were enumerated using a haemocytometer and diluted with MEM to give a cell density of 2×10^4/ml. Ninety-six well plates (Nunc) were seeded with 100 μl of this cell suspension per well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 hours to allow cell attachment. Subsequently, a range of concentrations of chemotherapeutic agent was added to the wells and plates were further incubated until the controls reached 80–90% confluence (typically 5–6 days). At the end of this period the medium plus the drug was decanted from the wells, the cells attached to the base of each well were washed with PBS and finally fixed with 10% formalin for 10 min. Cell growth was quantified using the crystal violet dye elution (CVDE) method [20]. Stained plates were read in a Titertek Multiscan MCC 340 plate reader at 570 nm and the results are expressed as a percentage of the growth attained by the controls. The CVDE method stains cells adhering to the base of the well so allowing the quantification of the extent of cell growth. All toxicity assays were performed in triplicate and a representative example is presented.

DNA gel electrophoresis

HEp-2 cells were harvested by trypsinization, washed and resuspended in PBS. Cells (1×10^6) were resuspended in lysis buffer (20 mM EDTA (Sigma), 0·8% (w:v) sodium lauryl sarcosinate, 100 mM Tris (pH 8·0)) (Merck) and 10 μl (10 mg ml^{-1}) RNase A (Boehringer Mannheim) and incubated at 37 °C for 18 hours. Proteinase K (1 mg ml^{-1}) (Boehringer Mannheim) was subsequently added and the samples incubated for a further 2 h at 50 °C. Prior to loading onto the gel 5 μl loading buffer (0·25% (w:v) bromophenol blue (Sigma), 50% (w:v) glycerol (Merck)) was added to the samples. Agarose gels (0·8% (w:v), BioRad) were prepared using 1 × TBE. Samples were loaded onto the gels and run at 55 v for 3·5 h. Lambda cl857 Sam 7 DNA digested with *Hin*dIII (BioRad) was used as DNA size standard. DNA was visualized under UV illumination following ethidium bromide staining.

FITC-Annexin V staining

FITC-Annexin V probe was obtained from Bendar Medsystems (BioWhittaker, UK). HEp-2 cells, previously exposed to the chemotherapeutic drugs for various periods, were resuspended in 95 μl of the binding buffer (10 mM Hepes/NaOH (pH 7·4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1·8 mM $CaCl_2$), and 10 μl of the undiluted probe was added [12]. Cells were incubated in the dark for 10 min at room temperature. Samples were subsequently diluted in 1 ml of binding buffer and analysed by flow cytometry on a FACScan (Becton Dickinson) using Lysis II software.

Adherence assay

The strains of *C. albicans* were grown to the stationary phase in YEPD at 37 °C and 200 rev min^{-1} in an orbital

incubator, harvested by centrifugation, washed twice with PBS and resuspended in PBS. HEp-2 cells for use in adherence assays were harvested using a cell scraper (Nunc), washed twice with PBS and resuspended in PBS. Yeast cells (5×10^7) and HEp-2 cells (1×10^5) were mixed in a final volume of 2 ml and incubated at 37 °C for 2 h. The mixture was subsequently smeared onto glass slides and allowed to air dry. This was heat fixed and stained with a 1·0% (w:v) crystal violet solution. The number of yeast cells adhering to each of 200 HEp-2 cells was enumerated microscopically per treatment. All adherence assays were performed in triplicate and results from representative assays are presented. The values for adherence represent the mean number of yeast cells adhering per HEp-2 cell ± SE of the mean. A Sigma-Stat package was used throughout this study for all statistical analyses. A Dunns *t*-test was performed to establish whether a significant difference existed between treatments and controls.

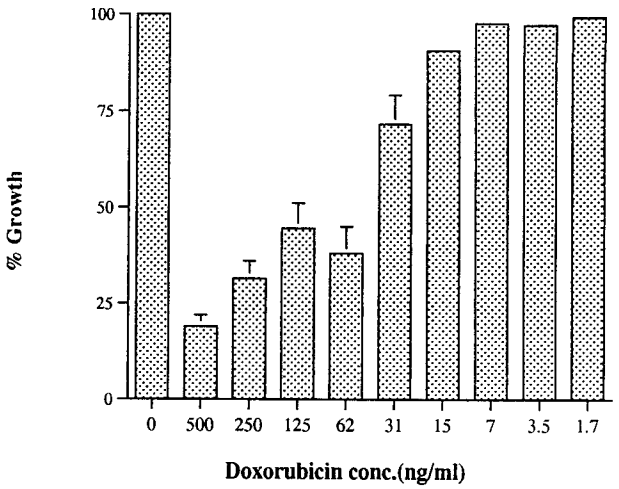

Fig. 1 Toxicity of doxorubicin to HEp-2 cells. HEp-2 cells were plated at a density of 2×10^4/ml in 96-well plates (Nunc). After 24 h incubation doxorubicin was added over the above concentration range. The extent of cell growth was determined after 5–6 days incubation using the CVDE method as described [20]. Growth is expressed as a percentage of that attained by the controls. The standard error is shown when greater than 2·5% of the mean.

Results

Measurement of relative toxicity of chemotherapeutic agents to HEp-2 cells

Assays to determine the relative toxicity of each of the three anticancer drugs to HEp-2 cells were performed so that levels sufficient to induce apoptosis rather than necrosis could be established. Ninety-six well plates were seeded with HEp-2 cells at a density of 2×10^3 per well. Cells were exposed to a range of concentrations of each chemotherapeutic agent and growth was quantified using the CVDE method as described. A representative sample of the results of such an assay using doxorubicin is presented (Fig. 1). The results from this assay allowed the identification of doxorubicin concentrations (15 ng ml^{-1} and 62 ng ml^{-1}) which induced cell death by apoptosis. In the case of methotrexate apoptosis was seen upon exposure to drug concentrations of 125 ng ml^{-1} and 250 mg ml^{-1} while cisplatin induced HEp-2 cell death by apoptosis at 400 ng ml^{-1} and 800 ng ml^{-1} (data not presented). In all cases the occurrence of apoptosis at the stated drug concentrations was confirmed by microscopic examination.

Induction of apoptosis in HEp-2 cells

HEp-2 cells were induced to die by apoptosis by exposure to the three chemotherapeutic agents at the previously established concentrations. Cells were exposed to the relevant concentrations of each drug, initially for 24 h, trypsinized and stained using FITC-Annexin V as described. Annexin V binding to PS has been used as a measure of the loss of membrane asymmetry associated with the onset of apoptosis [12]. The cells were analysed by flow cytometry on a FACScan and a representative set of results for doxorubicin treated cells is presented (Fig. 2). This figure shows the relative levels of fluorescence due to FITC-Annexin V binding to doxorubicin-treated and untreated HEp-2 cells. Cells which had been treated with doxorubicin demonstrate increased fluorescence as a result of the elevated levels of PS present on the outer leaflet of the plasma membrane, concomitant with the loss of membrane asymmetry. PS externalization, indicative of the loss of membrane asymmetry, was also evident when HEp-2 cells were treated with methotrexate or cisplatin for 24 h at the concentrations established previously.

Cultures of HEp-2 cells were exposed to doxorubicin (15 and 62 ng ml^{-1}) in a similar manner to that described above for periods of 24, 48 and 72 h. DNA was isolated from these cultures and separated by electrophoresis as described. The characteristic DNA fragmentation pattern, associated with the apoptotic-induced cleavage of nuclear DNA into nucleosome sized units, was only observed in cultures which had been exposed to drug for 72 h and not in those treated for 24 or 48 h (data not presented).

These results (detection of the loss of membrane asymmetry in apoptotic cells after 24 h and DNA fragmentation after 72 h exposure to chemotherapeutic drug) are consistent with the sequence of events in the apoptotic pathway in that the loss of membrane asymmetry is an early event [14,15] while the cleavage of DNA into discrete subunits occurs at a later stage in the process [8].

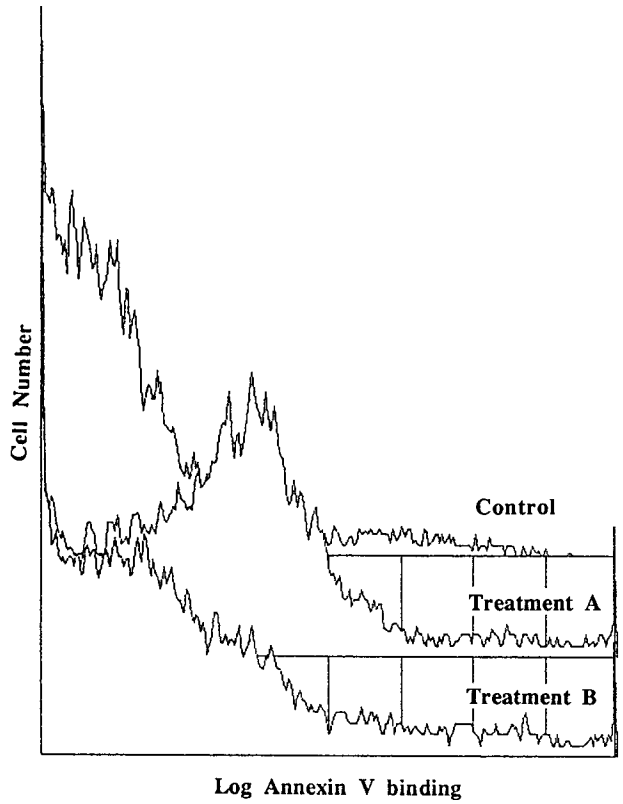

Fig. 2 FITC-Annexin V fluorescence of doxorubicin treated HEp-2 cells. Cultures of HEp-2 cells were exposed to 15 ng ml^{-1} (Treatment A) or 62 ng ml^{-1} (Treatment B) doxorubicin for 24 h. Cells were harvested, stained with FITC-Annexin V and analysed by flow cytometry using a Becton-Dickinson FACScan with Lysis II software. Ten thousand cells were analysed from each treatment.

Adherence of C. albicans *to HEp-2 cells in the early stages of apoptosis*

Subconfluent cultures of HEp-2 cells were exposed to each of the three anticancer drugs for 24 h and the induction of apoptosis was confirmed by the increased FITC-Annexin V fluorescence associated with PS externalization. Adherence assays were subsequently performed as described using three stains of *C. albicans* and HEp-2 cells in the early stages of apoptosis – as verified by the loss of membrane asymmetry.

The results of the series of adherence assays (Table 1) indicate that the overall susceptibility of HEp-2 cells to adherence by *C. albicans* decreased following treatment with concentrations of doxorubicin which had been shown to induce apoptosis: as indicated by the loss of membrane asymmetry. In the adherence assays which used *C. albicans* ATCC 44990 there is an almost 50% reduction ($P < 0.05$) in yeast adherence to HEp-2 cells treated with 62 ng ml^{-1} doxorubicin for 24 h. In the case of strains MEN and ATCC 10231 there is a 75% reduction ($P < 0.05$) and a 50% reduction ($P < 0.05$), respectively, in adherence to HEp-2 cells treated with this concentration of doxorubicin. HEp-2 cells exposed to 15 ng ml^{-1} doxorubicin also demonstrate a lower amount of adherence by the three *C. albicans* strains employed here (Table 1). These data demonstrate that treatment of HEp-2 cells with doxorubicin concentrations sufficient to induce apoptosis results in a decrease in the susceptibility of HEp-2 cells to adherence by the three strains of *C. albicans* used in this study.

Table 1 Mean number of *C. albicans* adhering per HEp-2 cell which had previously been exposed to doxorubicin

C. albicans strain	Doxorubicin concentration		
	Control	15 ng ml^{-1}	62 ng ml^{-1}
ATCC 44990	1·24 ± 0·06	0·83 ± 0·08	0·70 ± 0·07
MEN	1·13 ± 0·07	0·42 ± 0·06	0·30 ± 0·04
ATCC 10231	0·96 ± 0·07	0·69 ± 0·05	0·46 ± 0·04

HEp-2 cells were treated for 24 h with doxorubicin (15 and 62 ng ml^{-1}) and the induction of apoptosis was confirmed by the loss of membrane asymmetry as indicated by PS externalization. Adherence assays were performed as described. All values represent the mean number of yeast cells adhering per HEp-2 cell ± SE of the mean with 200 HEp-2 cells being enumerated per assay.

The reduction in adherence was not exclusively a feature of doxorubicin-induced membrane alterations associated with onset of apoptosis. Cultures of HEp-2 cells were exposed to methotrexate (125 and 250 ng ml^{-1}) and cisplatin (400 and 800 ng ml^{-1}) for periods of 24 h. Cells were subsequently harvested and stained with FITC-Annexin V to confirm the loss of membrane asymmetry. Adherence assays were conducted using HEp-2 cells treated with drug in a similar manner and cells of *C. albicans* ATCC 44990. The results from this series of assays indicated that treatment of HEp-2 cells with 250 ng ml^{-1} methotrexate or 800 ng ml^{-1} cisplatin for 24 h resulted in a reduction of 35% ($P < 0.05$) in the susceptibility of the HEp-2 cells to adherence by *C. albicans* (data not presented). The loss of membrane asymmetry, concomitant with the onset of apoptosis in HEp-2 cells, is sufficient to reduce the susceptibility of the HEp-2 cells to yeast adherence.

PS externalization in confluent cultures

By varying the cell density at which tissue culture flasks were seeded it proved possible to alter the kinetics of apoptosis induction and, hence, PS externalization. In this instance confluent cultures were exposed to doxorubicin (15 and 62 ng ml^{-1}) for periods of 18 and 40 h. Cells were harvested, stained with FITC-Annexin V and analysed for

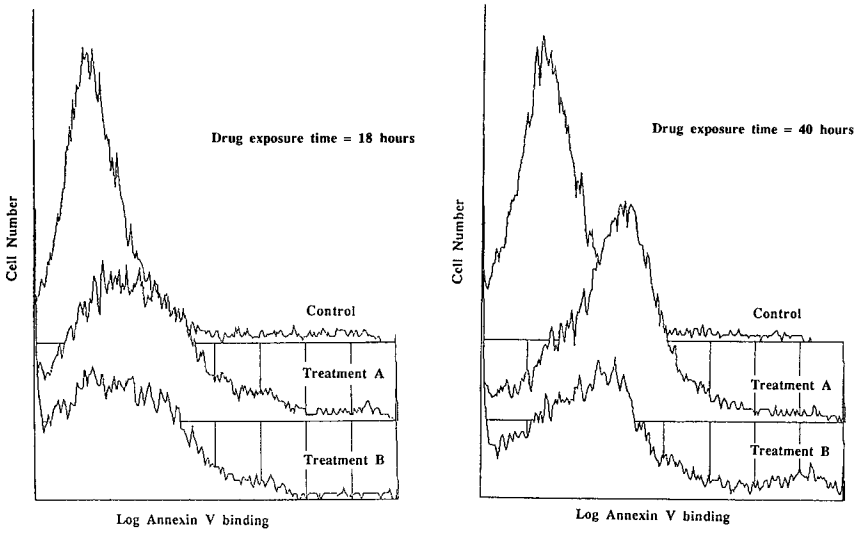

Fig. 3 FITC-Annexin V fluorescence of HEp-2 cells treated with doxorubicin. Confluent cultures of HEp-2 cells were exposed to 15 ng ml^{-1} (Treatment A) or 62 ng ml^{-1} (Treatment B) doxorubicin for 18 or 40 h. Cells were harvested, stained with FITC-Annexin V and analysed by flow cytometry using a FACScan (Becton Dickinson) equipped with Lysis II software. Ten thousand cells were analysed from each treatment.

PS externalization. Adherence assays were also performed using *C. albicans* ATCC 44990 and HEp-2 cells treated with drug for the above periods.

The result of these experiments indicate some PS externalization in cells exposed to doxorubicin for 18 h (Fig. 3). HEp-2 cells exposed to either of the doxorubicin concentrations demonstrate an increased FITC-Annexin V fluorescence compared with that evident for the control cells. This result indicates that some apoptosis is occurring in the population after 18 h incubation in the presence of doxorubicin. Incubation of cells for 40 h (Fig. 3) produced two clear regions of fluorescence: one associated with the control and the other with the cultures exposed to doxorubicin, indicating that membrane asymmetry has been lost in the drug-treated cultures as a consequence of the onset of apoptosis.

Adherence assays were performed with the HEp-2 cells which were treated with doxorubicin for the above time periods and cells of *C. albicans* ATCC 44990. The results of these assays (Table 2) indicate that after 18 h exposure to the drug, HEp-2 cells exhibit a lower susceptibility to *Candida* adherence than the control cells. Incubation in the presence of the drug for 40 h produced a substantial drop in adherence (in the case of each drug concentration adherence has dropped by approximately 50% ($P < 0.05$)). The reduction in susceptibility to yeast adherence is paralleled by the increased fluorescence associated with the Annexin V binding to PS present on the surface of the HEp-2 cells treated with doxorubicin (Fig. 3).

Discussion

Eukaryotic cell membranes usually restrict particular lipid species to one or other side of the bilayer thus creating an asymmetric distribution [21]. The loss of membrane asymmetry as indicated by PS externalization is an early indicator of the onset of apoptosis [11] and occurs without the loss of membrane integrity [12] and before cell shrinkage or nuclear condensation [13]. The aim of the work presented here was to establish whether the loss of membrane asymmetry, as determined by the externalization of PS, altered the susceptibility of HEp-2 cells to adherence by cells of *C. albicans*. Apoptosis was induced using three anticancer drugs and confirmed by the loss of membrane asymmetry (Fig. 2) and DNA cleavage. A reduction in the susceptibility of HEp-2 cells in the early stages of apoptosis to adherence by *C. albicans* was evident and this was apparent in confluent (Fig. 3 and Table 2) and subconfluent (Fig. 2 and Table 1) cultures in which apoptosis was induced by a number of anticancer agents. Although the three drugs used here have different modes of action [16–18], all induced apoptosis in HEp-2 cells

Table 2 Mean number of *C. albicans* ATCC 44990 adhering to HEp-2 cells from confluent cultures exposed to 15 ng ml^{-1} or 62 ng ml^{-1} doxorubicin for 18 or 40 h

	Exposure time to doxorubicin	
Doxorubicin conc.	18 h	40 h
Control	0·97 ± 0·09	0·79 ± 0·07
15 ng ml^{-1}	0·85 ± 0·09	0·38 ± 0·06
62 ng ml^{-1}	0·67 ± 0·08	0·41 ± 0·06

Confluent cultures of HEp-2 cells were treated with doxorubicin as described. After 18 or 40 h, cells were harvested and incubated with cells of *C. albicans* ATCC 44990 in adherence assays. All values represent the mean number of yeast adhering per HEp-2 cell ± SE of the mean with 200 HEp-2 cells being counted per treatment. Figure 3 indicates the relative levels of FITC-Annexin V fluorescence of HEp-2 cells used in these assays.

at the concentrations employed and the resulting cells displayed an increased resistance to yeast adherence.

One of the consequences of the loss of membrane asymmetry associated with apoptosis is an alteration in the nature of the surface to which the yeast cells adhere [10]. In apoptotic lymphocytes the membrane lipids are more loosely packed than normal cells, indicating an alteration in the outer leaflet of the plasma membrane [15]. PS is a negatively charged phospholipid which is normally located on the leaflet of the plasma membrane facing the cytosol [10]. The appearance of this phospholipid on the outer leaflet of cell membranes, as a result of the loss of membrane asymmetry, alters the surface charge and hydrophobicity of these surfaces [10,15] and such alterations could affect the non-specific adherence mechanisms of *C. albicans* [5,6]. The loss of membrane asymmetry also disrupts the normal configuration of the outer leaflet [10,13]. It is possible that ligands to which yeast cells normally bind may be 'hidden' or lost from the cell surface as a consequence of the loss of membrane asymmetry.

Although cancer patients receiving chemotherapy display an increased susceptibility to fungal infections, the work presented here indicates that HEp-2 cells induced to die by apoptosis using such drugs manifest an increased resistance to adherence by *C. albicans*. As adherence to host tissue is critical to the success of *C. albicans* as a pathogen [1,3,4], the increased resistance of apoptotic cells to adherence would appear to hinder fungal attachment to, and subsequent colonization of, host tissue. In addition to targeting neoplastic tissue, chemotherapeutic agents may also suppress the immune system which is a major factor in the increased incidence of fungal infections in such patients [22]. In the host, chemotherapeutic agents would appear to manifest a dual effect: cells induced to die by apoptosis using such drugs display an increased resistance to adherence by *C. albicans* while the host is more susceptible to invasive fungal diseases as a result of immunosuppression [23].

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