Mycopathologia **146:** 67–74, 1999.

Culture filtrates of *Aspergillus fumigatus* induce different modes of cell death in human cancer cell lines

Paul Daly[1], Steven Verhaegen[2], Martin Clynes[2] & Kevin Kavanagh[1]
[1]*Medical Mycology Unit, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland;*
[2]*National Cell and Tissue Culture Centre, BioResearch Ireland, Dublin City University, Glasnevin, Dublin 9, Ireland*

Received 2 March 1999; accepted in revised form 18 November 1999

Abstract

Aspergillus fumigatus culture filtrate (CF) has a potent cytotoxic effect on three human cancer cell lines (DLKP, A549 and HEp-2) and initiates cell death by apoptosis but the execution of the apoptotic process is incomplete. DLKP cells treated with *A. fumigatus* CF demonstrate features associated with apoptosis but cytoplasmic and nuclear fragmentation were not observed and cells ultimately underwent necrosis. The apoptotic process commenced in A549 and HEp-2 cells upon exposure to CF, cell shrinkage was observed but membrane blebbing and apoptotic body formation were not detected and detached cells died by necrosis. In contrast, extensive nuclear fragmentation and apoptotic body formation were evident in DLKP and A549 cells treated with anti-neoplastic agents. This work indicates that *A. fumigatus* CF is cytotoxic to cancer cells and can initiate apoptosis but that the complete apoptotic pathway is not followed.

Key words: apoptosis, *Aspergillus fumigatus*, cell death, culture filtrate(s), necrosis

Abbreviations: AV = Annexin V; CF = Culture Filtrate(s); PI = Propidium Iodide; TLVM = Time Lapse Videomicroscopy

Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen capable of colonising the lung or damaged airways of the bronchial tree in the immunocompromised host. Three classical presentations of aspergillosis are recognised: allergic, saphrophytic and invasive [1] but the mechanisms employed by *A. fumigatus* to colonise pulmonary tissue are poorly characterised [2].

Culture filtrates (CF) of *A. fumigatus* contain numerous mycotoxins with a variety of effects on mammalian cells which may facilitate fungal colonisation of host tissue [3]. *A. fumigatus* CF inhibit the action of complement [4], contain antiphagocytic factors that suppress local pulmonary defences [5], retard the ciliary beat frequency and destroy human respiratory epithelia [3]. The factors in CF responsible for cilioinhibition have been partially characterised as secondary metabolites and include gliotoxin, helvolic acid and fumagillin [6]. Gliotoxin inhibits macrophage function and adherence to plastic surfaces [7] and induces apoptotic cell death in macrophages [8], cells of the spleen [9] and the immune system [10], and in a murine fibroblastic cell line [11]. The induction of cell death, whether necrotic or apoptotic, by mycotoxins may precede and facilitate hyphal growth during fungal colonisation of the lung. Toxins of *A. fumigatus* have been implicated in the destruction of lung parenchyma and the penetration of blood vessels in angioinvasive aspergillosis [1].

The aim of the work presented here was to determine the response of human cancer cell lines following exposure to *A. fumigatus* CF. This would give an indication of the role of mycotoxins in facilitating fungal colonisation of the lung.

Materials and methods

Cell culture

The adherent human cancer cell lines used in this work were: DLKP (derived from a poorly differentiated human squamous lung cell carcinoma [12]), A549 (ATCC CCL 185, derived from a human lung carcinoma [13]) and HEp-2 (ATCC CCL 23, derived from an epidermoid carcinoma of the larynx [14]). Adherent cells were cultured in MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 5% (v/v) foetal calf serum (GIBCO Laboratories, Paisley, Scotland) and 4 mM L-glutamine (GIBCO). Cells were grown in 80 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C and 5% CO_2 in a humidified atmosphere and routinely subcultured by trypsinisation every 3–4 days.

Preparation of culture filtrate (CF)

A. fumigatus ATCC 13073 (originally isolated from a human pulmonary lesion) was obtained from the American Type Tissue Culture Collection (Rockville, MD) and grown on MEA plates (Blakeslee's formula; ATCC medium 325). Conidia were isolated using a 0.01% (v/v) Tween 80 (Sigma) solution, filtered through muslin to remove mycelial fragments, washed twice in phosphate buffered saline (PBS) and resuspended in 100 ml of MEM culture medium at a concentration of 1×10^6/ml. Fungal cultures were grown for 33 hours at 37 °C and 200 rpm in an orbital incubator. This had previously been shown to produce a toxic culture filtrate. Growth medium was decanted after centrifugation at 2300 g in a Beckman GS-6 centrifuge, filter sterilised with a 0.22 μm Acro-disc (Gelman Sciences, Ann Arbor, MI) and stored at −20 °C.

Experimental protocol

Twenty four hour old sub-confluent cultures (approximately 70% confluent) were used in all experiments. In each case culture medium was decanted and replaced with an equal volume of pre-warmed CF. Cells were re-incubated at 37 °C and 5% CO_2 in a humidified atmosphere for different periods and monitored by time lapse videomicroscopy, cytospin analysis, FAC Scan analysis or DNA agarose gel electrophoresis for alterations due to CF exposure. Control cells were incubated for the same periods in the absence of CF.

Cytospin analysis

Cells were harvested following exposure to CF for 24 hours and resuspended in PBS at a density of 1×10^6/ml. A 200 μl sample was spun for 3 minutes at 250 g using a Shandon Cytofuge 3 (Pittsburgh, PA). After air-drying, the samples were stained using Rapi-Diff II (Diakem, England), dried and mounted in DPX (BDH Chemical Co. Ltd, Dorset, England). Slides were analysed microscopically for the morphological characteristics of apoptosis or necrosis as described previously [15].

Time lapse videomicroscopy (TLVM)

Time lapse video microscopy (TLVM) was employed to monitor the morphological appearance of cells dying as a result of CF exposure [16]. TLVM was performed using a Nikon Diaphot inverted microscope equipped with phase-contrast optics (20× objective) linked to a Mitsubishi CCD-100E colour CCD camera via a 0.6× relay lens. Images were recorded in S-VHS onto a Mitsubishi HS-55600 video recorder with timelapse capabilities. The temperature of the culture vessel was maintained at 37 °C throughout.

FACScan analysis

Control cells and cells treated with CF for various periods were harvested by centrifugation for 5 minutes at 250 g in a Beckmann GS-6 centrifuge and washed with PBS. Cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and the cell number was adjusted to 2.5×10^5/ml. Undiluted Annexin V-FITC (5 μl) (Bender MedSystems, Bio Whittaker, UK) was added to 195 μl of the cell suspension and cells were incubated in the dark at room temperature for 15 minutes. Propidium iodide (Sigma) solution (40 μl of 100 μg/ml stock) and a further 160 μl of binding buffer were then added. FACS analysis was performed using flow cytometry on a FACScan (Becton Dickinson, San Jose, CA) using Lysis II software. Annexin V-FITC fluorescence is presented on the horizontal axis (FL1) and propidium iodide fluorescence on the vertical axis (FL2) of Figures 4 and 5. In each case the fluorescence of 10,000 cells was assessed.

DNA agarose gel electrophoresis

DNA was extracted from 1×10^6 cells by the addition of 20 μl of lysis buffer (2O mM EDTA, 0.8% (w/v)

sodium lauryl sarcosinate, 100 mMTris (pH 8.0)) and 10 μl of pre-boiled (1 mg/ml) RNase A (Boehringer Mannheim, Germany) and incubated for 18 hours at 37 °C. Proteinase K (Boehringer Mannheim, Germany) (10 μl of 10 mg/ml) was added to the cells which were incubated for 2 hours at 50 °C. Finally 5 μl of loading buffer (0.25% (w/v) bromophenol blue, 50% (w/v) glycerol) was added to the samples. Electrophoresis was performed at 55 V for 3.5 hours using a 1% (w/v) agarose gel in 1 × TBE solution. The DNA standard consisted of Lambda c1857 Sam 7 DNA digested with Hind III (BioRad, Hercules, CA). DNA was visualised under UV light following ethidium bromide staining.

Results

Microscopic analysis of cell death events

Thirty three hour old culture filtrates of *A. fumigatus* ATCC 13073 inhibit the growth of DLKP, A549 and HEp-2 cells (data not presented). The aim of the work presented here was to characterise the exact mode of death occurring in these cell lines following exposure to CF.

Medium was decanted from 24-hour old 70% confluent cultures and replaced with an equal volume of pre-warmed CF. Cultures were incubated at 37 °C and the response of cells was monitored with TLVM. DLKP cells began to detach from the the surface of the culture flask within 30 minutes of the addition of CF (Figure 1A) and after approximately 24 hours the majority of cells had rounded up, detached from the flask surface and began the characteristic blebbing of the plasma membrane associated with apoptosis (Figure 1B). Blebbing persisted for many hours but did not lead to the formation of apoptofic bodies as would be expected in the conventional apoptotic pathway [17]. Once blebbing ceased membrane integrity was lost indicating the occurrence of necrosis. Control cultures incubated for the same period showed no cell detachment after 24 hours incubation.

Cell detachment also commenced approximately 30 minutes after the addition of CF to sub-confluent cultures of A549. After 24 hours the majority of cells had detached and death was observed with the loss of cell membrane integrity as indicated by the loss of the phase-halo. No blebbing of the plasma membrane was observed. Control cells remained attached and formed an almost confluent layer. HEp-2 cells

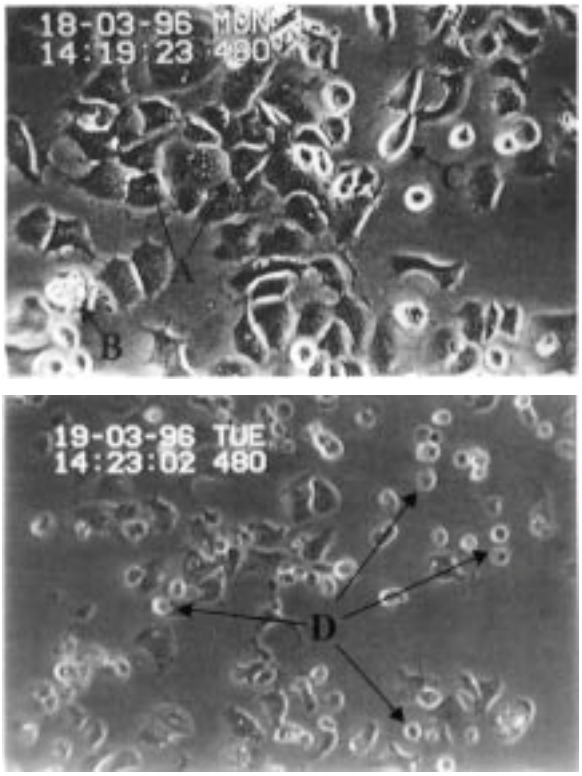

Figure 1. Microphotograph of DLKP cells 30 minutes and 24 hours after exposure to *A. fumigatus* CF. Cultures of DLKP cells were exposed to CF and the response monitored by TLVM. Figure 1A shows cells 30 minutes after the addition of CF. Note the normal adherent cells: (A) cells undergoing blebbing of the plasma membrane; (B) and cells retracting from the surface; (C) Figure 1B shows the culture after 24 hours. Note the rounded-up, detached cells in the field (D). (Magnification ×400.)

showed a similar pattern to that of the A549 cells with cells undergoing lysis, as indicated by the loss of the phase-halo, after 24 hours exposure to CF.

Cytospins of CF treated cells were analysed for the morphological characteristics of apoptosis or necrosis. Control DLKP cells showed a healthy appearance with no sign of nuclear condensation or fragmentation into apoptotic bodies (Figure 2A). Cells treated for 24 hours with CF demonstrated dramatic nuclear and physical alterations including cell shrinkage and nuclear condensation (Figure 2B). Extensive nuclear fragmentation did not occur and the presence of apoptotic bodies was not observed in these cells. It was apparent that some cells had lysed. In contrast DLKP cells treated with the anti-neoplastic agent Taxol undergo extensive nuclear fragmentation and apoptotic body formation (Verhaegen and Clynes, unpublished observations).

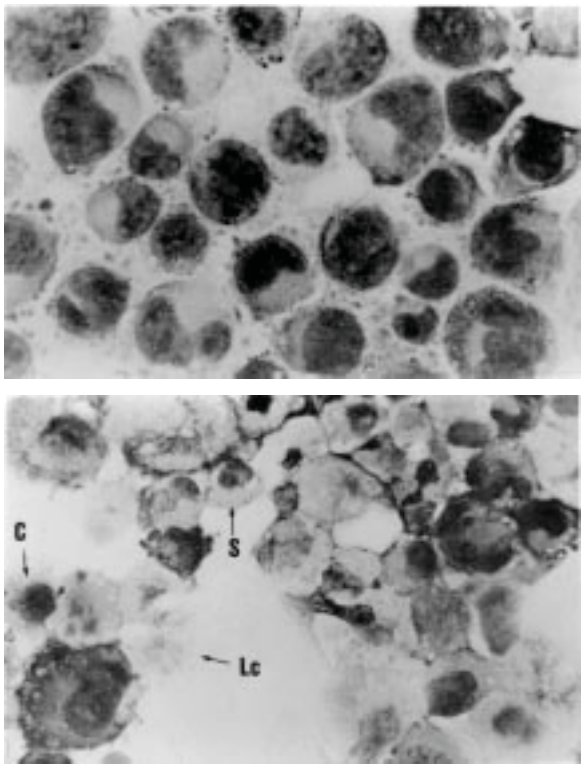

Figure 2. Morphological features of control and CF-treated DLKP cells. Sub-confluent DLKP cultures were treated with *A. fumigatus* CF for 24 hours and then prepared for microscopic analysis as described. Note the healthy appearance of normal cells (Figure 2A). Figure 2B shows features of DLKP cells treated with CF for 24 hours. Note nuclear and cytoplasmic alterations including cell shrinkage (S) and nuclear condensation (C). Apoptotic bodies are absent. Lysed cells (Lc) are visible. (Magnification × 1100.)

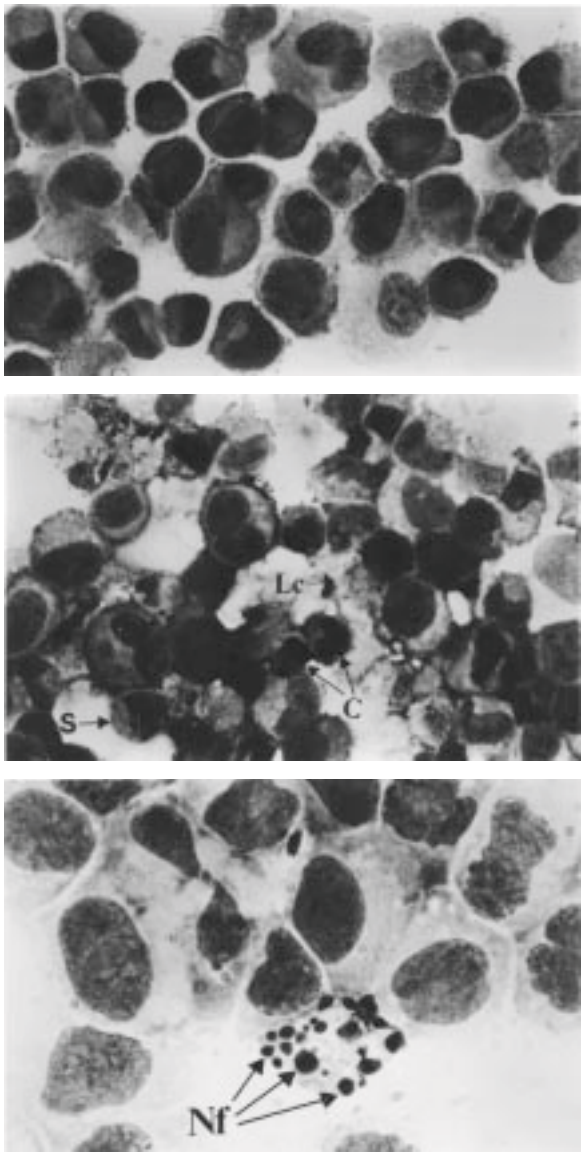

Figure 3. Morphological features of A549 cells. Cytospins of control, CF-treated and etoposide-treated A549 cells. Figure 3A shows normal cells. Figure 3B shows cells exposed to CF for 24 hours. Note cell shrinkage (S), nuclear condensation (C) and evidence of cell lysis (Lc). Figure 3C shows A549 cells after exposure to 100 μg/ml etoposide for 24 hours, note extensive nuclear fragmentation (Nf). (Magnification × 1100.)

CF produced only cell shrinkage and nuclear condensation in A549 cells (Figures 3A and 3B) and neither nuclear fragmentation nor apoptotic body formation was detected. In contrast, exposure of A549 cells to the anti-neoplastic agent Etoposide (Bristol Myers Pharmaceuticals, UK) at a concentration of 100 μg/ml for 24 hours resulted in extensive nuclear fragmentation and apoptotic body formation (Figure 3C). In the case of HEp-2 cells cytospin analysis revealed that exposure to CF for 24 hours resulted in cell shrinkage, nuclear condensation and ultimately lysis. Neither nuclear fragmentation nor apoptotic body formation was observed.

Flow cytometric analysis

Annexin V-FITC (AV) can be used in association with propidium iodide (P1) to assess the relative levels of apoptosis and necrosis in cell populations [18]. Apoptotic cells appear in the lower right area of the FACS-plots while necrotic cells register in the upper right region. Normal cells are recorded in the lower left quadrangle. Based upon AV and P1 double staining, after 24 hours in the presence of CF the level of apoptosis in DLKP cells was 17.90% while the level of necrotic cells was 18.76% (Figure 4B). In contrast,

the 24 hour control culture had 7.27% of cells apoptotic and 2.29% necrotic (Figure 4A). After 48 hours 49.10% of cells were necrotic (Figure 4D) while the equivalent control culture showed that 11.86% of cells were apoptotic and 3.03% were necrotic (Figure 4C).

Following 24 hours exposure to CF 47.86% of A549 cells appeared necrotic while 10.89% demonstrated a staining pattern indicative of apoptosis (Figure 5B). The comparable control culture showed low levels of apoptosis and necrosis (Figure 5A). After 48 hours 81.81% of CF-treated cells were necrotic and 13.03% appeared apoptotic (Figure 5D) while in the comparable control culture 4.42% of cells demonstrated a staining pattern indicative of apoptosis and 1.66% appeared necrotic (Figure 5C). Flow cytometric analysis of HEp-2 cells showed the occurrence of a similar cell death pathway as seen in DLKP, i.e., a predominance of necrotic cells after 48 hours exposure to CF.

Detection of DNA fragmentation

Agarose gel electrophoresis can be used to distinguish the different forms of cells death, i.e., the internucleosomal fragmentation of cellular DNA following the induction of apoptosis appears as a DNA 'ladder' [17]. DNA was extracted from control cells and cells treated with CF for 24, 48 or 72 hours as described and separated by agarose gel electrophoresis. The results reveal that CF was incapable of inducing sufficient levels of DNA fragmentation in DLKP cell populations after 24 hours to give a pronounced ladder pattern (Figure 6). Fragmentation of DNA into the characteristic ladder pattern associated with apoptosis was observed after 48 and 72 hours exposure to CF (note the 'ladder' patterns in lanes 3 and 4 of Figure 6). Control populations of DLKP incubated for the same periods in the absence of CF did not yield a DNA 'ladder' fragmentation pattern. A549 cells showed a weak DNA banding pattern irrespective of the length of the incubation period.

Discussion

The work presented in this paper demonstrates that *A. fumigatus* CF is capable of initiating apoptotic cell death in adherent human cancer cell lines but that the process terminates before the completion of the full apoptotic pathway. Apoptosis is a form of cell death which is biochemically and morphologically distinct from necrosis [17]. It may occur as part of the normal developmental processes involved in tissue formation or as a result of a variety of cellular insults or stimuli [19]. During apoptosis the dying cell actively participates in its own demise by following a specific sequence of events which include detachment from its neighbours or tissue culture substrate, cell shrinkage and membrane blebbing. Nuclear events include condensation of the chromatin and extensive internucleosomal cleavage of cellular DNA which is visible as a ladder of fragments of multiples of approximately 180 base pairs in size when DNA from apoptotic cells is analysed by agarose gel electrophoresis [17]. Blebbing of the plasma membrane is followed by fragmentation of the cell into neatly packaged apoptotic bodies which may be engulfed by neighbouring cells or phagocytes without evoking an inflammatory response. The necrotic form of cell death generally follows severe injury [20]. During necrosis, the cell volume increases and membrane integrity is lost early in the process. As a consequence, the contents of the cell are released into the immediate environment which may result in further damage to surrounding cells and an inflammatory response [17].

A. fumigatus CF caused cell detachment and inhibited the growth of the cell lines used in this work. Time-lapse video microscopy analysis of CF treated DLKP cells showed numerous features associated with apoptosis including cell shrinkage and blebbing of the plasma membrane (Figure 1B). Cytospins of CF treated cells revealed a decrease in cell size, but also nuclear condensation and cell lysis. However at no stage was apoptotic body formation detected (Figure 2B). FAC Scan analysis using AV and P1 double staining of DLKP cells treated with CF for 24 hours showed features associated with apoptosis, i.e., AV staining of external plasma membrane phosphatidylserine, but also the presence of necrotic cells (Figure 4B). In the work presented here deviations from the 'conventional' apoptotic pathway have been observed. DLKP cells treated with CF detached from the flask surface, decreased in size and underwent membrane blebbing. Fragmentation of DNA using conventional gel electrophoresis was also detected (Figure 6). Apoptotic body formation did not occur and many of the apoptotic cells finally lost their membrane integrity – a phenomenon closely associated with necrosis, suggesting an incomplete execution of the apoptotic process.

Cell death in the A549 cell line following treatment with CF showed a number of differences to that which occurs in DLKP. TLVM failed to show blebbing of

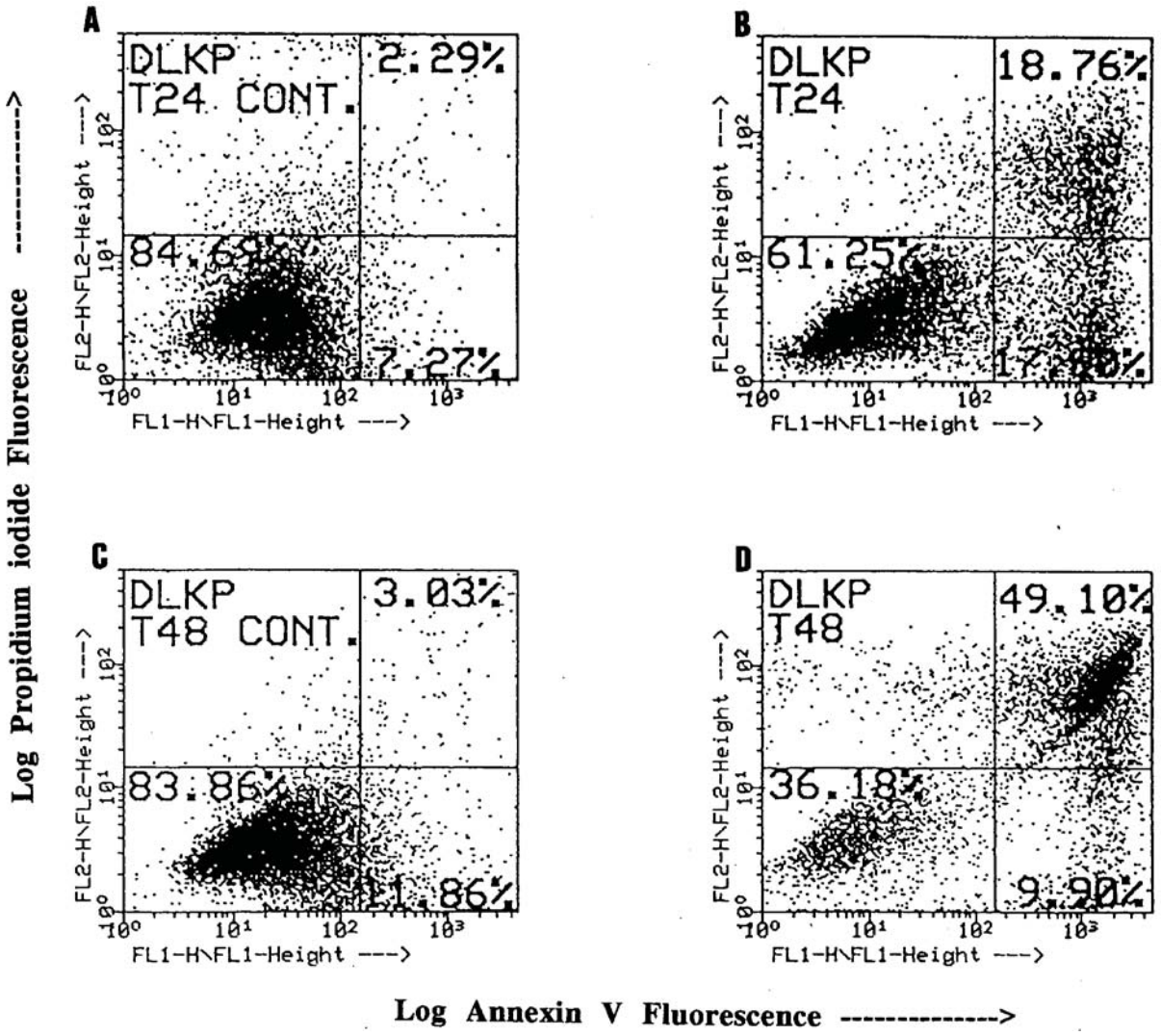

Figure 4. Flow cytometric analysis using AV and P1 staining of DLKP cells. Twenty four hour old sub-confluent cultures of DLKP cells were exposed to CF for 24 and 48 hours, harvested as described and analysed by flow cytometry for alterations in AV and P1 fluorescence (Figures 4B and 4D). Controls were cultured for the same periods in the absence of CF (Figures 4A and 4C).

the plasma membrane and only weak fragmentation of DNA could be detected using gel electrophoresis. In contrast, the form of cell death evident in this cell line following exposure to etoposide resulted in apoptosis terminating in extensive nuclear fragmentation and the formation of apoptotic bodies (Figure 3C).

HEp-2 cells showed a decrease in cell size and increased AV staining when treated with CF but appear to die by necrosis. There was no evidence of fragmentation of DNA into the characteristic 'ladder' pattern of apoptosis in this cell line, indicating cell death via necrosis rather than apoptosis.

Since CF contains many components, some of which remain to be characterised [6], some component(s) may force the abandonment of the apoptotic pathway prior to the commencement of the latter stages of the process. Fumonisin B1, a mycotoxin produced by *Fusarium moniliforme*, has been shown to inhibit apoptosis in P388 and U937 cells [21]. Since apoptosis, but not necrosis, is ATP dependent it is possible that some component(s) in CF may deplete intracellular ATP levels thus pushing the cells towards necrosis [22]. DLKP and A549 cells are capable of completing the full apoptotic pathway and forming apoptotic bodies when apoptosis is induced by anti-

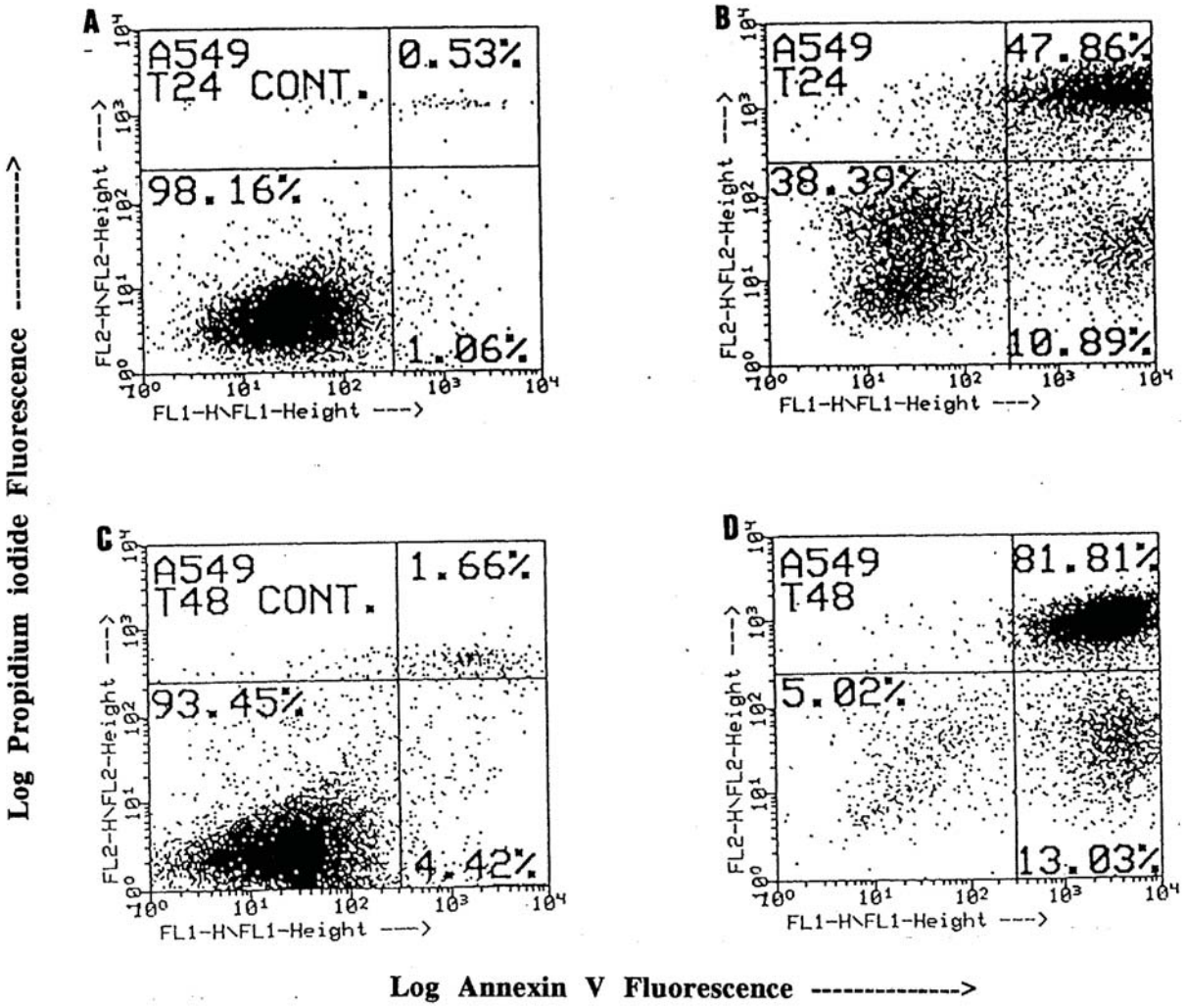

Figure 5. Flow cytometric analysis of AV and P1 staining of A549 cells. Twenty four hour old sub-confluent cultures of A549 cells were exposed to CF for 24 and 48 hours, harvested as described and analysed by flow cytometry for alterations in AV and P1 fluorescence (Figures 5B and 5D). Controls were cultured for the same periods in the absence of CF (Figures 5A and 5C).

neoplastic agents indicating that the type of cell death observed here following CF treatment is not an inherent feature of the cell lines. The forms of cell death described here contrast with that recorded in murine fibroblasts exposed to gliotoxin (a component of *A. fumigatus* CF) where DNA fragmentation and the formation of apoptotic bodies occurs [11]. Gliotoxin induces apoptosis, as determined by gel electrophoresis and flow cytometry, in macrophages [8] and spleen cells [9] but it is unclear whether morphological studies were performed to ascertain if these processes terminated in apoptotic body formation or necrosis.

This work demonstrates that *A. fumigatus* CF-induced cell death assumes a variety of forms in the human cancer cell lines examined here. One cell line (DLKP) demonstrated many of the stages of apoptosis (cell shrinkage, membrane blebbing, DNA fragmentation) before undergoing necrosis. A549 and HEp-2 cells appeared to begin the apoptotic process (demonstrated cell shrinkage as determined by cytospin analysis and flow cytometry) but then lysed. The induction of cell death by mycotoxins during colonisation of the lung facilitates hyphal growth and tissue penetration [1]. There remains the possibility that *A. fumigatus* CF may contain some component(s)

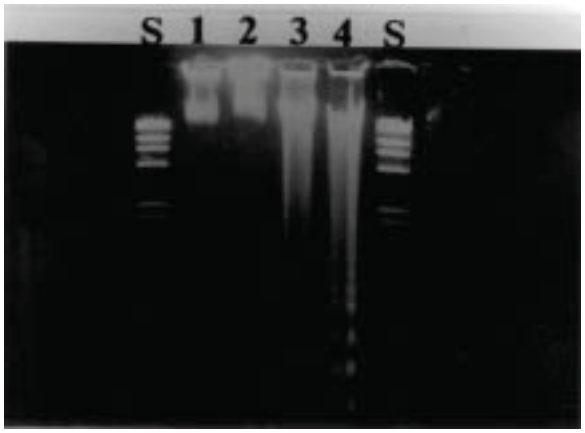

Figure 6. Agarose gel electrophoresis of DNA extracted from control and CF-treated DLKP cells. DNA was extracted from control cells and cells exposed to CF for various periods and analysed by agarose gel electrophoresis. Lane 1 – control cells; Lane 2 cells treated with CF for 24 hours; Lane 3 – cells treated with CF for 48 hours; Lane 4 – cells treated with CF for 72 hours. S represents DNA size marker with weights, in descending order, of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb.

that circumvent the full apoptotic pathway before the formation of apoptotic bodies.

References

1. Fraser RS. Pulmonary aspergillosis: Pathologic and pathogenetic features. Pathol Annul 1993; 28: 231–277.
2. Hogan LH, Klein BS, Levitz SM. Virulence factors of medically important fungi. Clin Microbiol Rev 1996; 9: 469–488.
3. Amitani R, Murayama T, Nawada R, Lee WJ, Niimi A, Suzuki K, Tanaka E, Kuze F. *Aspergillus* culture filtrates and sputum sols from patients with pulmonary aspergillosis cause damage to human respiratory ciliated epithelium in vitro. Eur Respir J 1995; 8: 1681–1687.
4. Washburn RG, Hammer CH, Bennett JE. Inhibition of complement by culture supernatants of *Aspergillus fumigatus*. J Infect Dis 1986; 154: 944–951.
5. Murayama T, Amitani R, Ikegami Y, Nawada Y, Lee WJ, Kuze F. Suppressive effects of *Aspergillus fumigatus* culture filtrates on human alveolar macrophages and polymorphonuclear leucocytes. Eur Respir J 1996; 9: 293–300.
6. Amitani R, Taylor G, Elezis E, Jones CL, Mitchell J, Kuze F, Cole PJ, Wilson R. Purification and characterisation of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect Immum 1995; 63: 3266–3271.
7. Eichner RD, Al Salami M, Wood PR, Mullbacher A. The effect of gliotoxin upon macrophage function. Int J Immunophamac 1986; 8: 789–797.
8. Waring P. DNA fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceeded by raised inositol triphosphate levels. J Biol Chem 1990; 265: 14476–14480.
9. Braithwaite AW, Eichner RD, Waring P, Mullbacher A. The immunomodulating agent gliotoxin causes genomic DNA fragmentation. Molec Immunol 1987; 24: 47–55.
10. Sutton P, Newcombe NR, Waring P, Mullbacher A. In vivo immunosuppressive activity of gliotoxin, a metabolite produced by human pathogenic fungi. Infect Immun 1994; 62: 1192–1198.
11. Piva TJ. Gliotoxin induces apoptosis in mouse L929 fibroblast cells. Biochem Mol Biolint 1994; 33: 411–419.
12. Law E, Gilvarry V, Grant G, Gregory B, Clynes M. Cytogenic comparison of two poorly differentiated human lung cell carcinoma cell lines. Cancer Genet Cytogenet 1992; 59: 111–118.
13. Lieber M, Smith B, Szakal A, Rees WN, Todaro G. A continuous tumour-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 1976; 17: 62–70.
14. Moore AE, Sabachewsky L, Toolan HW. Culture characteristics of four permanent lines of human cancer cells. Cancer Res 1955; 15: 598–603.
15. Lennon SV, Martin SJ, Cotter TG. Dose dependent induction of apoptosis in human tumour cell lines by widely divergent stimuli. Cell Prolif 1991; 24: 203–214.
16. Verhaegen, S. Microscopical study of cell death via apoptosis. Microscop Anal 1998; 5–7.
17. Cohen JJ. Overview: Mechanisms of apoptosis. Immunol Today 1993; 14: 126–130.
18. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger, C. A novel assay for apoptosis: How cytometric detection of phosphatidylserine expression on early apoptofic cells using fluorescein labelled Annexin V. J. Immunol Meths 1995.184: 39–51.
19. Cotter TG, Lennon SV, Glynn JG, Martin SJ. Cell death via apoptosis and its relationship to growth. Development and differentiation of both tumour and normal cells. Anticancer Res 1990; 10: 1153–1160.
20. Collins, RJ, Harmon BV, Gobe GC, Kerr JFR. Internucleosomal DNA clevage should not be the sole criterion for identifying apoptosis. Int J Radiat Biol 1992; 61: 451–453.
21. Bose R, Verheji M, Halmovitz-Friedman A, Scotto K, Fuks Z, Kolesnick K. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. Cell 1995; 82: 405–414.
22. Eguchi Y, Shimizu S, Tsujimoto T. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 1997; 57: 1835–1840.

Address for Correspondence: Dr. Kevin Kavanagh, Medical Mycology Unit, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland
Tel.: +353-1-708 3859; Fax: +353-1-708 3845;
E-mail: kevin.kavanagh@may.ie