***Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid**

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Recent evidence has revealed the occurrence of an apoptotic phenotype in *Saccharomyces cerevisiae* that is inducible with oxidative stress. Here, exposure of *S. cerevisiae* to 20–200 mM acetic acid for 200 min at pH 3.0 resulted in cell death. Yeast mortality induced by 120–200 mM acid was not inhibited by cycloheximide and was accompanied by ultrastructural alterations typical of necrosis. In contrast, alterations associated with cell death induced by 20–80 mM acetic acid included: (i) cycloheximide-inhibitable chromatin condensation along the nuclear envelope; (ii) exposure of phosphatidylserine on the surface of the cytoplasmic membrane, revealed by the FITC-annexin V reaction; and (iii) the occurrence of DNA strand breaks, demonstrated by the TUNEL assay. These results show that a programmed cell death process sharing common features with an apoptotic phenotype can be induced by acetic acid in *S. cerevisiae*. This observation raises the possibility of this mode of cell death being more generalized in yeasts than previously considered and extended to cell death induced by other stress agents.

Keywords: active cell death, apoptosis, yeast, acid stress

INTRODUCTION

In animal cells, apoptosis, necrosis and atypical cell death have been identified as distinct forms of cell death (Darzynkiewicz *et al.*, 1997). A cell triggered to undergo apoptosis activates a widely described cascade of molecular events, which results in a programmed cell suicide. The occurrence of programmed cell death in unicellular organisms was initially discarded based on the altruistic role attributed to such a cell death process in multicellular organisms. However, cell suicide responses have already been reported in prokaryotic and unicellular eukaryotic cells (Matsuyama *et al.*, 1999). It has also been recognized that the sacrifice of individuals of a population of unicellular organisms can be advantageous to the rest of the population. Hence, by analogy with apoptosis, the term ‘phenoptosis’ has been proposed to designate programmed cell death of an organism, either multi- or unicellular (Skulachev, 1999a, 2000).

Abbreviations: PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labelling.

The reported mechanisms underlying programmed cell death in prokaryotes are different from those involved in apoptosis of animal cells. Examples include mechanisms involved in programmed cell death of plasmid-free segregants, avoiding in this way the survival of bacteria unable to retain the plasmid (Yarmolinsky, 1995; Naito *et al.*, 1995), and programmed cell death of phage-infected *Escherichia coli*, with the prevention of virus spreading among the bacterial population (Matsuyama *et al.*, 1999; Skulachev, 1999a, 2000). Moreover, programmed cell death in bacteria also plays an important role in developmental processes, such as lysis of the mother cell during sporulation of *Bacillus subtilis* and lysis of vegetative cells in fruiting body formation of *Myxococcus xanthus* (Lewis, 2000). In addition, programmed cell death has been reported in unicellular eukaryotic organisms (Ameisen, 1996). The yeast *Saccharomyces cerevisiae* has been used mainly as a tool to study human apoptosis regulatory proteins and it is not generally accepted to have an endogenous programmed cell death process. However, evidence indicating the presence of some basic features characteristic of an apoptotic phenotype in *S. cerevisiae* was recently reported (Madeo *et al.*, 1997; Ligr *et al.*, 1998). The

expression of the anti-apoptotic protein Bcl-2 in *S. cerevisiae* provides antioxidant protection and delays natural death (Longo *et al.*, 1997). Furthermore, it was recently shown in *S. cerevisiae* that depletion of glutathione or exposure to low external doses of H_2O_2 triggered the cell into apoptosis, whereas depletion of reactive oxygen species (ROS) or hypoxia prevented apoptosis (Madeo *et al.*, 1999). In addition, an intracellular accumulation of ROS was detected in the cell cycle mutant *cdc48*^{S565G} of *S. cerevisiae* and in yeast cells expressing mammalian Bax. These results allowed the identification of ROS production as a key cellular event common to the known scenarios of apoptosis in yeast and animal cells (Madeo *et al.*, 1999). Recently, it was suggested that the Rad9 protein of *Schizosaccharomyces pombe* could be a member of the Bcl-2 protein family, being the first protein of this family identified in yeast (Komatsu *et al.*, 2000). The above evidence has suggested that a programmed cell death process occurs in yeasts and shares some conserved aspects with metazoan apoptosis. Nevertheless, yeast cell death processes are far from being clarified. Characteristic features of cell death patterns in yeast need to be identified in order to provide some insights into the cell death mode(s) in this unicellular eukaryote.

Acetic acid is a normal end product of the alcoholic fermentation carried out by *S. cerevisiae*. This compound is not metabolized by glucose-repressed yeast cells and enters the cell in the undissociated form by simple diffusion. Inside the cell, the acid dissociates and if the extracellular pH is lower than the intracellular pH, this will lead to intracellular acidification, anion accumulation and inhibition of the metabolic cell activity, namely fermentation/respiration (Leão & van Uden, 1986; Cássio *et al.*, 1987; Pampulha & Loureiro, 1989). Moreover, it was shown that in *S. cerevisiae*, under certain conditions, acetic acid compromises cell viability and ultimately results in two types of cell death, high and low enthalpy (Pinto *et al.*, 1989). Previous studies from our laboratory have focused on the assessment by flow cytometry of cell structural and functional changes induced by acetic acid in *S. cerevisiae* and *Zygosaccharomyces bailii* (Ludovico, 1999; Prudêncio *et al.*, 1998). However, the process by which the yeast cell dies when injured by acetic acid is unknown.

The aim of the present study was to identify morphological, structural and functional cellular markers that would allow the characterization of the mode(s) of cell death induced by acetic acid in *S. cerevisiae*.

METHODS

Micro-organism and growth conditions. The yeast *Saccharomyces cerevisiae* W303-1A (MATa *ade2 leu2 his3 trp1 ura3*) was used. This organism was maintained on YEPD agar slants containing glucose (2%, w/v), yeast extract (1%, w/v), peptone (2%, w/v) and agar (2%, w/v). In experiments, the yeast cells were subcultured in liquid YEPD medium. The growth experiments were performed in 250 ml flasks containing a 2:1 ratio of air to liquid phase, and incubated on a

mechanical shaker (150 r.p.m.) at 26 °C. Cells were grown to mid-exponential phase.

Treatments with acetic acid and inhibition of protein synthesis. Exponential-phase cells were harvested and suspended (10^7 cells ml⁻¹) in YEPD (pH 3.0, set with HCl) containing 0, 20, 40, 80, 120, 160 and 200 mM acetic acid. The treatments were carried out for 200 min at 26 °C with magnetic stirring (100 r.p.m.). The inhibition of protein synthesis was performed by adding 100 µg cycloheximide (Merck) ml⁻¹ (Barnett *et al.*, 2000) to the cell suspensions at the same time as the different acetic acid concentrations tested. Cycloheximide at this concentration was not cytotoxic after 200 min incubation, as assessed by c.f.u. counts. Viability was determined by c.f.u. counts after 2 d incubation at 26 °C on YEPD agar plates. No further colonies appeared after that incubation period. In all the above experiments, the extracellular pH did not change during the incubations.

Transmission electron microscopy analysis. Cells from different treatment conditions were washed with phosphate/magnesium buffer (40 mM K₂HPO₄/KH₂PO₄, pH 6.5, 0.5 mM MgCl₂), suspended in 2.5% (v/v) glutaraldehyde in 40 mM phosphate/magnesium buffer, pH 6.5, and fixed overnight at 4 °C. After fixation, the cells were rinsed twice in 0.1 M phosphate/citrate buffer (pH 5.8) and suspended in this buffer containing 10 U lyticase (Boehringer Mannheim) ml⁻¹ for about 90 min at 37 °C, to digest the cell wall. After cell wall digestion of the pre-fixed yeast cells, protoplasts were washed and postfixed with 2% (w/v) osmium tetroxide (2 h) followed by 30 min incubation with 1% (w/v) aqueous uranyl acetate. Dehydration was performed as described by Byers & Goetsch (1991) for embedding vegetatively grown yeast cells. After 100% ethanol washes, the samples were transferred to 100% propylene oxide, and infiltrated with 50% (v/v) propylene oxide and 50% (v/v) Epon (TAAB Laboratories) for 30 min and with 100% Epon overnight. Cells were transferred to gelatin capsules with 100% Epon and incubated at 60 °C for 48 h before cutting thin sections and staining with uranyl acetate and lead acetate. Micrographs were taken with a Zeiss EM 10C electron microscope. For quantitative assessment of yeast cells with condensed chromatin, at least 50 yeast cells with nuclear profiles were counted per sample.

Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) and propidium iodide staining. DNA strand breaks were demonstrated by TUNEL with the 'In Situ Cell Death Detection Kit, Fluorescein', from Boehringer Mannheim. This technique labels free 3'-OH termini with FITC-labelled deoxyuridine triphosphate (dUTP), which was detected by epifluorescence microscopy. Yeast cells were fixed with 3.7% (v/v) formaldehyde as described by Madeo *et al.* (1999) and cell walls were digested with lyticase (as above, for 105 min). Cytospins of the cell suspensions were made using a Shandon Cytospin 2 cytocentrifuge, operating at 1500 r.p.m. for 5 min. The slides were rinsed with PBS, incubated in permeabilization solution (0.1%, v/v, Triton X-100 and 0.1%, w/v, sodium citrate) for 2 min on ice, rinsed twice with PBS and incubated with 10 µl TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase and FITC-dUTP, for 60 min at 37 °C. Finally the slides were rinsed three times with PBS and a coverslip was mounted with a drop of anti-fading agent Vectashield (Molecular Probes) and with 2 µl of a propidium iodide (PI; Molecular Probes) working solution (50 µg ml⁻¹ in Tris buffer (10 mM, pH 7.0) with MgCl₂ (5 mM) and RNase (0.5 µg ml⁻¹). Observations were carried out using a Zeiss Axioskop epifluorescence microscope equipped with a HBO-100 mercury lamp, filter set 40 (BP360/51, BP485/17, BP560/18) from Zeiss, excitation filter

BP 450-490, beam splitter FT510 and emission filter LP520. Images were acquired with a Spot 2 camera (Diagnostic Instruments). For the quantitative assessment of TUNEL staining, 50–700 cells were counted per sample.

Annexin V staining. Phosphatidylserine exposure was detected by an FITC-coupled annexin V reaction with the ApoAlert Annexin V Apoptosis kit (CLONTECH Laboratories), essentially as described by Madeo *et al.* (1999). Cells were harvested and washed with sorbitol buffer (1.2 M sorbitol, 0.5 mM MgCl₂, 35 mM K₂HPO₄, pH 6.8). Cell walls were digested with 15 U lyticase (Sigma) ml⁻¹ and 2% (v/v) glusulase (NEE-154 Glusulase; NEN) in sorbitol buffer for about 30 min at 28 °C; digestion with the two enzymes was monitored by phase-contrast microscopy in order to prevent damage to the unfixed protoplasts. Cells were then washed twice with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂; CLONTECH Laboratories) containing 1.2 M sorbitol. To 38 µl cell suspension in binding/sorbitol buffer were added 2 µl annexin V (20 µg ml⁻¹) and 2 µl of a PI working solution (as described for the TUNEL reaction) and incubated for 20 min at room temperature. The cells were then washed and resuspended in binding/sorbitol buffer. Finally the slides with coverslips were mounted with the cell suspensions. Microscope settings and image acquisitions were as described above for the TUNEL technique. For quantitative assessment of annexin V–PI staining, at least 150 yeast cells were counted per sample.

Reproducibility of the results. All the experiments were repeated at least three times. The data reported for c.f.u. counts are mean values and standard deviations. Quantitative data for annexin V and TUNEL staining and for chromatin condensation are from one representative experiment; while absolute data were not comparable in these experiments performed on different days, the trends described were fully consistent in the independent experiments.

RESULTS

Exposure of *S. cerevisiae* strain W303-1A to acetic acid at different concentrations (20–200 mM), pH 3.0, resulted in cell death (Fig. 1). The percentage of survival, estimated by c.f.u. counts after 200 min treatment, decreased with increasing concentrations of the acid in

Fig. 1. Relative survival (% of c.f.u. on YEPD agar plates; 100% corresponds to the number of c.f.u. at time zero) of *S. cerevisiae* incubated for 200 min with acetic acid in the absence (◆) or presence (■) of cycloheximide. Vertical error bars represent standard deviations.

Fig. 2. Electron micrographs of exponentially growing cells of *S. cerevisiae*. (a) Untreated control, (b) cell treated with 40 mM and (c) cell treated with 160 mM acetic acid for 200 min. N, Nucleus. Bar, 1 µm.

the extracellular medium. In order to address whether cell death induced by acetic acid treatment was an active process, the same experiments were carried out in the presence of cycloheximide. The presence of this inhibitor of protein synthesis attenuated the toxic effects of acetic acid and enhanced the survival percentage (Fig. 1). However, the protective effect of cycloheximide was

Table 1. Cycloheximide inhibits chromatin condensation induced in *S. cerevisiae* by exposure to 20–120 mM acetic acid for 200 min

The percentage of yeast cell profiles with chromatin condensation along the nuclear envelope was determined by counting at least 50 cell profiles per sample. The values in samples exposed to acetic acid in the presence of 100 µg cycloheximide ml⁻¹ are given in parentheses. ND, Not determined.

[Acetic acid] (mM)	Chromatin condensation (%)
0	0 (0)
20	ND
40	78.0 (4.8)
80	73.6 (30.0)
120	9.7 (1.2)
160	0 (0)
200	0 (0)

observed only for the lower acid concentrations; for concentrations higher than 120 mM the survival percentages were not significantly increased by the drug. In fact, a very high mortality was seen in yeasts exposed to 120 mM acid regardless of the presence of cycloheximide (99.86 and 99.90 %, with and without the inhibitor, respectively). This indicates that the cell death process induced at the higher concentrations was not dependent on protein synthesis.

Subsequently, it was attempted to characterize further the type of cycloheximide-inhibitable cell death induced by acetic acid at the lower acid concentrations. Thus, samples of cell suspension treated with the acid were harvested and assayed for different apoptotic markers, namely chromatin condensation along the nuclear envelope, exposure of phosphatidylserine at the outer surface of the plasma membrane and formation of DNA strand breaks.

Extensive chromatin condensation along the nuclear envelope in cells treated with 20–80 mM acetic acid (shown in Fig. 2b for 40 mM) was revealed by quantitative transmission electron microscopy analysis (Table 1), whereas nuclei of untreated cells were homogeneous in shape and density (Fig. 2a). Only a low

Table 2. Characterization of *S. cerevisiae* cells (%) according to FITC-coupled annexin V and PI staining after exposure to 20–120 mM acetic acid for 200 min

Cell category*	[Acetic acid] (mM)			
	20	40	80	120
Annexin V (–), PI (–)	86.6	47.1	10.1	0.0
Annexin V (+), PI (–)	12.5	40.4	40.7	14.7
Annexin V (+), PI (+)	0.9	12.5	43.5	41.1
Annexin V (–), PI (+)	0.0	0.0	5.7	44.2

* Cell category as defined in Results.

percentage of cells treated with 120 mM acid, and no cells treated with 160 or 200 mM, exhibited chromatin condensation (Table 1). Consistent with the enhancement by cycloheximide of the survival of *S. cerevisiae* to 40–80 mM acetic acid treatment, a decrease in the number of cells with chromatin condensation along the nuclear envelope was evident in the presence of cycloheximide (Table 1).

However, in many yeast cells treated with 120 mM acetic acid and in most cells treated with 160 mM, alterations typical of necrosis were observed by electron microscopy, namely an extensive cell disorganization with most intracellular structures destroyed (shown in Fig. 2c for 160 mM).

In yeasts, as in mammalian cells, phosphatidylserine has an asymmetric distribution in the lipid bilayer of the cytoplasmic membrane (Cerbón & Calderón, 1991). The exposure of phosphatidylserine at the outer surface of the cytoplasmic membrane occurs at the early stages of apoptosis (Martin *et al.*, 1995) when membrane integrity is still retained. Therefore, with the technique used in the present study, which includes simultaneous staining with FITC-coupled annexin V and the membrane impermeant fluorochrome PI, those early apoptotic cells only stain green, from FITC, indicating the presence of phosphatidylserine at the outer surface of the plasma membrane [annexin V (+), PI (–)], shown in Fig. 3a for 20 mM acetic acid]. Numbers of annexin V (+), PI (–) cells were maximal at 40–80 mM acetic acid (Table 2). Yeast cells in more advanced apoptotic stages, or necrotic cells, stain green and red due to the inability

Fig. 3. Annexin V–PI staining of *S. cerevisiae* exponentially growing cells exposed to 20 (a) or 120 (b) mM acetic acid for 200 min. The cell in Fig. 3(a) is stained only in green by annexin V [annexin V (+)], and not in red [PI (–)], indicating that it is in an early stage of apoptosis. In contrast, the cells in Fig. 3(b) are stained in both green [annexin V (+)] and red [PI (+)], indicating that they are in advanced apoptosis or in necrosis.

Fig. 4. Left panel: TUNEL reaction after 200 min of treatment of exponentially growing *S. cerevisiae* cells with 0 (a), 40 (b) or 120 (c) mM acetic acid. Right panel: phase-contrast of the fields in the left panel.

of the cell membrane to exclude PI [annexin V (+), PI (+), shown in Fig. 3b for 120 mM acid]. Numbers of annexin V (+), PI (+) cells were maximal at 80 and 120 mM acetic acid (Table 2). In the 120 mM sample, most of the annexin-V-negative cells were PI-positive [annexin V (–), PI (+)]. Untreated cells did not exhibit a positive annexin V reaction or PI staining [annexin V (–), PI (–)].

Treatment of *S. cerevisiae* with 20, 40 and 80 mM acetic acid resulted in a TUNEL-positive phenotype (yellow nuclear fluorescence as the result of superimposition of green and red fluorescence due to simultaneous staining with TUNEL and PI, as shown in Fig. 4b for 40 mM), indicating the occurrence of DNA strand breaks. The percentage of cells displaying a positive TUNEL staining increased with the acetic acid concentration (19.0% and 51.5% of TUNEL-positive cells were counted in preparations treated with 40 and 80 mM acid, respectively). With 120 mM acetic acid, a few cells (5.7%) showed a positive TUNEL reaction (Fig. 4c). Incubation with acetic acid concentrations from 160 to 200 mM resulted in no detectable TUNEL staining. Control cells maintained in YEPD medium, pH 3.0, for 200 min in the absence of acetic acid showed a negative TUNEL staining (Fig. 4a).

For reasons not yet completely understood, cells exposed to cycloheximide (with or without acetic acid) and then stained by the annexin V or TUNEL reactions displayed a bright yellow fluorescent background that made it impossible to analyse the effect of the inhibitor on the two apoptotic markers.

Preliminary results have shown that the apoptotic markers referred to above are present, in a concentration-dependent way, in samples exposed to 20–120 mM acetic acid for time periods shorter than the 200 min used in the present study.

DISCUSSION

Although inhibitors of protein synthesis may, in some cases, prevent the anti-apoptotic cascade (Skulachev, 1999b), it has been reported that in many cell types apoptosis can be prevented by the inhibition of protein synthesis (Hiraoka *et al.*, 1997; Sánchez *et al.*, 1997). Recently, it was described in *S. cerevisiae* that cycloheximide prevented the apoptotic effects of H₂O₂ (Madedo *et al.*, 1999). In the present study, it is shown that, in this species, exposure to 20–80 mM acetic acid for 200 min induces a programmed cell death pathway that requires the functionality of the protein synthesis machinery. This conclusion was based on the observation that cycloheximide inhibited cell death induced by acetic acid at that low concentration range but not at the higher acid concentrations. Although with 120 mM it was still possible to detect this inhibition, it occurred in a very small sub-population, more than 99.86% of the cells remaining non-viable regardless of the presence of cycloheximide.

The mode of programmed cell death under acetic acid stress conditions was further characterized by the examination of cell death markers that are typical of apoptosis. The evidence obtained indicates that acetic

acid, like H_2O_2 (Madeo *et al.*, 1999), can trigger an apoptotic phenotype in *S. cerevisiae*. The following concentration-dependent changes were observed after treatment with acetic acid: chromatin condensation along the nuclear envelope, exposure of phosphatidylserine at the outer surface of the yeast cytoplasmic membrane and formation of DNA strand breaks. The nuclear ultrastructural changes were significantly reduced when the treatment was carried out in the presence of cycloheximide, reinforcing our interpretation about the active nature of cell death induced by the lower concentration of acetic acid.

Even though a very small population with apoptotic markers was detected at 120 mM, the present results suggest that a non-active (necrotic) process is the predominant cell death mechanism in samples exposed for 200 min to acetic acid at concentrations above 80 mM. This interpretation is based on the following points: (i) mortality at 120 mM (or more) is not significantly inhibited by cycloheximide; (ii) the percentage of cells with apoptotic markers, induced by increasing concentrations of acid, increases with treatments up to 80 mM acid and then decreases; (iii) the percentage of cells with ultrastructural alterations typical of necrosis is highest and very extensive in samples treated with 120–160 mM acid. An apoptotic or necrotic phenotype was also reported for *S. cerevisiae* exposed for 200 min to low or high concentrations of H_2O_2 , respectively (Madeo *et al.*, 1999).

Our data regarding the structural/morphological markers of apoptosis showed that the proportion of yeast cells with chromatin condensation induced by 40 mM acetic acid was higher than the proportion of cells exhibiting surface exposure of phosphatidylserine [annexin V (+), PI (–)] and DNA cleavage (TUNEL-positive). This observation indicates that, in our model, nuclear chromatin alteration precedes both phosphatidylserine exposures at the cytoplasmic membrane surface and significant endonuclease DNA cleavage. Similar observations have been reported in other models of apoptosis, i.e. chromatin condensation preceding DNA cleavage (Sun *et al.*, 1994) and phosphatidylserine exposure (Darzynkiewicz *et al.*, 1997).

The yeast *S. cerevisiae* has been used as a host to express proteins of the Bcl-2 family and hence to study the mechanisms underlying apoptosis in higher eukaryotes. Up to now, only one instance of an active cell death process in yeasts, independent of heterologous expression of pro-apoptotic proteins, has been reported, namely programmed cell death due to exposure of *S. cerevisiae* to H_2O_2 (Madeo *et al.*, 1999). In the present work, we have shown that the apoptotic phenotype in *S. cerevisiae* can also be induced by acetic acid, pointing to the possibility that this mode of cell death may be more generalized in yeasts and extended to other stress agents. It is known that ROS play a central role in the regulation of apoptosis at various levels (Madeo *et al.*, 1999). Further studies are in progress in order to evaluate ROS

production in *S. cerevisiae* under the conditions where programmed cell death induced by acetic acid was detected. This would show if commitment of *S. cerevisiae* to a programmed cell death process in response to acetic acid is also mediated through a ROS-dependent apoptotic pathway.

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