Vacuole-mitochondrial cross-talk during apoptosis in yeast: a model for understanding lysosome-mitochondria-mediated apoptosis in mammals

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Abstract
The yeast apoptosis field emerged with the finding that key components of the apoptotic machinery are conserved in these simple eukaryotes. Thus it became possible to exploit these genetically tractable organisms to improve our understanding of the intricate mechanisms of cell death in higher eukaryotes and of severe human diseases associated with apoptosis dysfunctions. Early on, it was recognized that a mitochondria-mediated apoptotic pathway showing similarities to the mammalian intrinsic pathway was conserved in yeast. Recently, lysosomes have also emerged as central players in mammalian apoptosis. Following LMP (lysosomal membrane permeabilization), lysosomal proteases such as cathepsins B, D and L are released into the cytosol and can trigger a mitochondrial apoptotic cascade. CatD (cathepsin D) can also have anti-apoptotic effects in some cellular types and specific contexts. Nonetheless, the mechanisms underlying LMP and the specific role of cathepsins after their release into the cytosol remain poorly understood. We have recently shown that yeast vacuoles, membrane-bound acidic organelles, which share many similarities to plant vacuoles and mammalian lysosomes, are also involved in the regulation of apoptosis and that the vacular protease Pep4p, orthologue of the human CatD, is released from the vacuole into the cytosol in response to acetic acid. Here, we discuss how the conservation of cell-death regulation mechanisms in yeast by the lysosome-like organelle and mitochondria may provide new insights into the understanding of the complex interplay between the mitochondria and lysosome-mediated signalling routes during mammalian apoptosis.

Lysosomes: role in cell death and cross-talk with mitochondria
In the last decade, it has been demonstrated that organelles and proteases other than mitochondria and caspases, such as lysosomes and cathepsins, are also engaged in the regulation of mammalian cell-death processes (for a revision, see [1–3]). Cellular responses to different death stimuli may entail mitochondrial membrane permeabilization associated with the release of pro-apoptotic factors into the cytosol, as well as LMP (lysosomal membrane permeabilization) coupled with the release of cathepsins. LMP can trigger distinct cell-death processes involving mitochondria-mediated apoptosis (either caspase-dependent or -independent), as well as necrosis.

Key words: apoptosis, cathepsin D, lysosome, mitochondrion, Pep4p, vacuole.
Abbreviations used: AAD, ADF/AYF carrier; ANF, adenosine nucleotide translocator; CatD, cathepsin D; CRC, colorectal carcinoma; cyt c, cytochrome c; EGF, enhanced green fluorescent protein; LMP, lysosomal membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization; PCD, programmed cell death; ROS, reactive oxygen species; sCET, short-chain fatty acid.

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The cell-death process is determined by the nature of the lethal stimulus, the released cathepsins, the levels of endogenous cathepsin inhibitors and the extent of LMP. Extensive collapse of lysosomes causes necrosis, whereas moderate permeabilization of lysosomes leads to apoptosis [4]. Depending on the lethal stimuli and cell context, LMP can occur early in the apoptotic process and be critical for activation of the signalling cascade or can take place later in the apoptotic process and contribute to amplification of the death signal. The involvement of the lysosomal cathepsins B, L and D in the regulation of apoptosis has been shown in various cellular models using both genetic manipulations and pharmacological inhibitors. CatD (cathepsin D) has emerged as a central player in the apoptotic response, although its role is cell type and context dependent. Indeed, it is generally accepted that CatD is overexpressed and plays an important role in cancer cells [5]. Pro-CatD outside the cells induces proliferation, angiogenesis, invasion and metastasis [6]. Additionally, it was demonstrated that inhibition of CatD with pepstatin A induces caspase-dependent apoptosis in neuroblastoma cell lines [7] and that overexpression of
in intracellular CatD in mouse xenografts using rat-derived cell lines inhibits apoptosis [5]. It was also reported that CatD down-regulation sensitizes neuroblastoma cells to doxorubicin-induced apoptosis, while the opposite effect is observed for CatD overexpression [8]. In contrast, CatD mediates cyt c (cytochrome c) release and caspase activation in staurosporine-induced apoptosis in human fibroblasts [9]. It is therefore apparent that CatD can have opposite roles in apoptosis and that the lysosome is intrinsically connected to apoptosis through LMP. Nevertheless, the molecular mechanisms underlying CatD release and LMP and their role in connecting lysosomal to mitochondrial membrane permeabilization are not completely understood. Moreover, several data suggest that some CatD substrates have not yet been identified [4]. The observation that distinct molecules [e.g. caspas, ROS (reactive oxygen species), Bax and Bid] can act both upstream and downstream of LMP, further denotes a complex cell death signalling network.

In the following sections, we describe how the yeast vacuole plays a similar role to lysosomes in the regulation of apoptosis. Moreover, we discuss how understanding the involvement of the vacuole and of Pep4p, the orthologue of the human CatD, in a mitochondria-dependent apoptotic pathway during yeast cell death will be exceedingly useful in the elucidation of some yet unclarified roles of lysosomes and CatD in mammalian apoptosis.

**Yeast apoptosis: role of the vacuole and the yeast CatD**

The first report on the involvement of the vacuole in yeast apoptosis concerns the translocation of the vacuolar protease Pep4p, the orthologue of the human CatD, into the cytosol during H$_2$O$_2$-induced apoptosis [10]. It was found that, in an early phase of cell death, ROS levels and nuclear permeability increase while cell viability decreases. In a later phase, the vacuolar membrane becomes permeable and provides access of the protease to nucleoporin substrates. Similar to the partial LMP observed during mammalian apoptosis, the release of Pep4p–EGFP (enhanced green fluorescent protein) from the vacuole is not linked to a rupture of the vacuolar membrane, as evidenced by a vacuolar lumen morphologically distinct from the cytosol. However, PEP4-deleted cells are not protected from H$_2$O$_2$-induced cell death. This may be explained by the fact that migration of Pep4p out of vacuoles and nucleoporin degradation occur only after the cells are unviable. Release of Pep4p from the vacuolar compartment is also observed in an End3p-deficient mutant displaying actin cytoskeleton stabilization-induced apoptosis [11]. However, a role for this protease in actin-stabilized dying cells was not ascertained by the authors. Another study also documented the involvement of the vacuole in yeast apoptosis [12]. Deletion of class C vacuolar protein-sorting genes results in drastically enhanced sensitivity of yeast to treatment with acetic acid and leads to a necrotic death, whereas death is mainly apoptotic in the wild-type strain. These results indicate that a functional vacuole is required for a regulated cell-death process through apoptosis [12].

We have recently found that Pep4p is released from the vacuole into the cytosol in cells undergoing acetic acid-induced apoptosis [13]. Transmission electron microscopy analysis of the dying cells showed that vacuolar membrane integrity is preserved and plasma membrane integrity is maintained. Therefore Pep4p release seems to involve partial permeabilization of the vacuolar membrane rather than an extensive permeabilization typical of necrotic death. Taken together, these results suggest that Pep4p could have a role in apoptotic cell death similar to that of mammalian CatD. Unexpectedly, we observed that deletion of PEP4 confers higher susceptibility to acetic acid [13], suggesting a function in cell protection rather than in the execution of cell death. Sustaining this hypothesis, cells overexpressing Pep4p display a higher resistance to acetic acid.

Different alterations in mitochondrial structure and function occurring during yeast apoptosis have been identified. These changes include reduction in cristae number and mitochondrial swelling (see [14]), a transient mitochondrial hyperpolarization followed by depolarization, production of ROS, decrease in cytochrome oxidase activity and MOMP (mitochondrial outer membrane permeabilization), with concomitant release of cyt c and yeast AIF (Aifp) and EndoG (endonuclease G) (Nuc1p) [15–17]. Yeast orthologues of some of the mammalian permeability transition pore components were found to be involved in MOMP and cyt c release. While deletion of POR1 (coding for the yeast VDAC (voltage-dependent anion channel)) enhances apoptosis triggered by acetic acid, CPR3 (coding for mitochondrial cyclophilin) deletion has no effect. In contrast, absence of AAC (ADP/ATP carrier) proteins, yeast orthologues of the adenine nucleotide transporter, protects cells exposed to acetic acid and impairs MOMP and cyt c release [18]. Mitochondrial proteins involved in fission/fusion, namely, Fis1p, Dnm1p and Mdv1p [19] have also been implicated in the execution of the yeast apoptotic programme induced by acetic acid. It has also been shown that, similarly to different mammalian apoptotic scenarios, acetic acid induces mitochondrial fragmentation and degradation.

Pep4p-deficient cells, as well as the wild-type strain, exhibit mitochondrial dysfunction but are delayed in mitochondrial degradation during acid-induced apoptosis [13]. On the other hand, Pep4p overexpression slightly enhances mitochondrial degradation under the same conditions. Therefore the process of removing damaged mitochondria apparently has a protective role in acetic acid-treated cells, although it is probably not the only factor affecting cell viability. In yeast, selective removal of mitochondria was reported following heterologous expression of Bax [20], mitochondrial dysfunctions [21], osmotic swelling [22] and in stationary phase cells [23]. Removal of mitochondria is not always dependent on the autophagic machinery [20], and the outcome in terms of survival obtained by blocking mitochondrial degradation varies with the stimulus used (for a review, see [24]). Notably, autophagy is not activated during acetic...
Cross-talk between mitochondria and the vacuole in yeast-PCD induced by acetic acid

Current working model illustrating the link between the effect of acetic acid on mitochondria and vacuoles, which involves mitochondrial membrane permeabilization, associated with the release of cyt c, Aifp and Muc1p into the cytosol, and vacuole membrane permeabilization, coupled with the release of the yeast CatD orthologue, Pep4p.

Acetic acid-induced apoptosis and therefore removal of mitochondria cannot be achieved through this process. Therefore the involvement of the vacuole and Pep4p in mitochondrial degradation is autophagy independent, although the precise mechanism is unknown. It is, however, apparent that this process also involves non-vacuolar proteins. Indeed, AAC-deficient cells show a decrease in mitochondrial degradation in response to acetic acid as well, and are not defective in Pep4p release. Therefore AAC proteins seem to affect mitochondrial degradation at a step downstream of Pep4p release, possibly triggering degradation through their involvement in mitochondrial permeabilization. Accordingly, our results indicate that the sensitization of cells to acetic acid by deletion of PEP4 is dependent on AAC proteins, again suggesting these proteins act downstream of Pep4p in the apoptotic cascade (F. Azevedo, S. Chaves, B. Johansson, M.J. Sousa and M. Côte-Real, unpublished work).

Taken together, the aforementioned observations suggest that vacuole and mitochondria destabilization, as measured by Pep4p and cyt c release respectively, are events in the cell-death cascade. Even though CatD, the mammalian orthologue of Pep4p, was shown to have a role in cell death by triggering mitochondrial dysfunction and subsequent release of mitochondrial proteins, some studies have shown an inhibitory role for CatD in apoptosis. Nonetheless, a role for CatD in mitochondrial degradation in mammals has not been assessed so far. Since autophagy is not active in cells undergoing acetic acid-induced apoptosis, vacuolar membrane permeabilization associated with the release of Pep4p may act as an alternative mitochondrial degradation process. The cytosolic acidification induced by acetic acid, associated with inhibition of autophagy, may favour the activity of Pep4p after its release from the vacuole. We are currently investigating how this protease is released from the vacuole and involved in mitochondrial degradation. The finding that both mitochondrial AAC proteins and the vacuolar Pep4p interfere with mitochondrial degradation suggests a complex regulation and interplay between mitochondria and the vacuole in yeast-PCD (programmed cell death; Figure 1).

There are still several questions that need to be addressed to achieve a more complete picture of the communication between mitochondria and the vacuole, as well as of the acetic acid cellular targets and signalling pathways. Indeed, we still do not know whether acetic acid targets the vacuole to trigger membrane permeabilization and Pep4p release, which in turn signal to mitochondria, or whether it acts directly at both the vacuole and mitochondrion, and initiates a communication between these two organelles (Figure 1). Purified and physiologically active vacuoles should provide a good model system to study the role of these organelles in yeast apoptosis in response to a lethal stimulus. With this aim, we have isolated yeast vacuoles and characterized the purified fraction with regard to their structural and functional integrity. Phase-contrast and fluorescence microscopy show that a purified vacuolar fraction can be obtained (Figure 2). Moreover, the isolated vacuoles are structural and functionally intact (A. Pedras, H. Gerós and M. Côte-Real, unpublished work), validating the possibility of using isolated organelles to clarify the mechanisms underlying the release of Pep4p and how it relates to alterations in vacuole function.

The parallel between acetic acid-induced pathways in yeast and mammalian cells

Acetic acid has also been shown to induce apoptosis in a mammalian cell model. It has been demonstrated that the SCFAs (short-chain fatty acids) acetic and propionic acids produced by dietary propionibacteria in the human intestine induce cell death in CRC (colorectal carcinoma) cell lines.
by a mitochondria-dependent apoptotic pathway [25,26] as described for yeast cells [15,27]: SCFA-induced nuclei shrinkage, chromatin condensation, nuclei fragmentation into apoptotic bodies and activation of pro-caspase 3. Moreover, it was shown that the mitochondrial dysfunctions induced by SCFAs in CRC cells are similar to those observed in yeast, and can also be partially inhibited by expression of anti-apoptotic members of the Bcl-2 protein family [25,28]. Jan et al. [25] determined that the ANT (adenine nucleotide translocator), a putative component of the mammalian PTPC (permeability transition pore complex), was a potential SCFA target. Likewise, AAC proteins, the yeast orthologues of ANT, are targets in the acetic acid-induced apoptosis pathway [18]. Recently, we also found that acetate induces LMP and the release of CatD in CRC cells undergoing apoptosis (C. Marques, M. Côrte-Real, O. Coutinho and A. Preto, unpublished work). The observation that acetate triggers a mitochondrial apoptotic pathway involving the vacuole/lysosome in both yeast and CRC cells further supports the use of the yeast model system to provide insights into enhanced understanding of the function of lysosomes in cell death and their cross-talk with mitochondria. Moreover, the clues afforded by the yeast model will allow designing specific assays with mammalian cell lines to confirm the hypothesis emerging from the yeast data.

Concluding remarks

While relevant differences in cellular processes exist between yeast and mammalian cells, the conservation of a vacuolar and mitochondrial role in yeast apoptosis offers new perspectives for an enhanced understanding of the roles of lysosomes and mitochondria in mammalian apoptosis. Indeed, the approaches with yeast, harbouring a lysosome-like vacuole, are more efficient in providing new hints on the lysosomal cell-death components and the cross-talk with mitochondria than the less accessible and more complex mammalian cells. The importance of lysosomes in mammalian cell death is increasingly apparent, and thus our studies further reinforce the use of yeast as a valuable model of PCD. Moreover, our findings identifying a new function for Pep4p, and AAC proteins in yeast may unveil a novel role for their orthologues in mammalian cells. Finally, as lysosomal dysfunction is associated with different human pathologies, a deeper understanding of the role of lysosomes in apoptosis regulation and of the mechanisms underlying LMP and the connection to MOMP may unravel novel attractive targets for the development of apoptosis-based therapies.

Funding

This work was supported by the ‘fundaç~ao para a Ci~encia e Tecnologia’ project [grant numbers FCOMP-01-0124-FEDER-007047 and PTDC/AGR-ALI/100636/2008].

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Received 31 May 2011
doi:10.1042/BST0391533