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Cellular catabolism in apoptosis: DNA degradation and endonuclease activation

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Abstract. Recent research has focused on identifying the biochemical events associated with the apoptotic process. These include specific degradation of the chromatin which was described by Wyllie in 1980 [1], with the report of the appearance of discretely sized DNA fragments from apoptotic rat thymocytes. The fragments corresponded in size to strands of DNA that were cleaved at internucleosomal regions and create a 'ladder pattern' when electrophoresed on an agarose gel. Because of its near universality, internucleosomal DNA degradation is considered a diagnostic hallmark of cells undergoing apoptosis. It is of great interest to identify the enzymes involved, and some of the candidates will be discussed.
Key words. Apoptosis; cyclophilin; DNA degradation; NUC18; endonuclease.

Introduction

The events comprising apoptosis were first described morphologically, based on microscopic studies of tissue sections at various developmental stages or following the removal or addition of hormones that lead to tissue involution. More recent research has focused on identifying the biochemical events associated with the apoptotic process. Because of the different responses of individual cell types to various apoptotic inducers, it is prudent to use both morphological and biochemical characteristics to ascertain whether a cell is undergoing apoptosis. Such a combined approach should allow us to understand how a cell carries out this catabolic event.

Electron microscopic studies revealed several distinct stages of apoptotic death [2]. In the early stage of apoptosis, the cell begins to shrink and pull away from surrounding cells. The plasma membrane loses its microvilli and initially gains a smooth exterior [2, 3]. This process obviously reflects a mechanism to isolate the apoptotic cell from neighbours, preventing the spread of death. Cell shrinkage is also associated with a decrease in the size of the nucleus and condensation of the chromatin. The condensed chromatin moves to the periphery of the nucleus and the nucleolus disintegrates.

A still later aspect of apoptosis is a continuation of the degradation of nuclear and cytoplasmic components. This can occur either in apoptotic bodies that have already been phagocytosed or in noningested apoptotic bodies, in which the process is sometimes referred to as secondary necrosis [4].

Biochemical characteristics of apoptosis

Once the foundations for defining apoptosis morphologically were established, the biochemical events underlying this death process were investigated by many laboratories around the world. Ironically, these events have proven more difficult to define because of the wide variety of systems studied and the simple fact that one contaminates the process with degraded cellular constituents if apoptosis is allowed to go to completion. However, there are several common biochemical features of catabolism shared by cells undergoing apoptosis. These include specific degradation of the chromatin, a general decrease in protein concentration, alterations in membrane structure, and induction and inhibition of certain genes. In this review we will focus our attention on the activation of endonucleases and DNA degradation during apoptosis.

DNA degradation

Specific degradation of the chromatin DNA is one of the most thoroughly studied apoptotic events [5–9]. It was described by Wyllie in 1980 [1], with the report of the appearance of discretely sized DNA fragments from apoptotic rat thymocytes. The fragments corresponded in size to strands of DNA that were cleaved at internucleosomal regions and create a 'ladder pattern' when electrophoresed on an agarose gel. With few exceptions [10, 11] apoptotic cells display this pattern of chromosomal degradation. Because of its near universality, internucleosomal DNA degradation is considered a diagnostic hallmark of cells undergoing apoptosis. Cells that do not appear to have internucleosomal DNA degradation were investigated with a new type of electrophoretic technology, the pulsed field gel electrophoresis system, which revealed a second pattern of apoptotic DNA fragmentation. In this case, the DNA is fragmented into much larger pieces that are approximately 50 kb in length. These larger pieces of DNA are proposed to arise from release of loops of DNA. A

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model of chromosome packing describes binding of the chromatin DNA to the nuclear scaffold at approximately 50 kb intervals so that the remaining unbound portion of the DNA loops out from the protein scaffold (the 'rosette' structure) [12]. Cleavage at regularly spaced intervals, such as the scaffold attachment regions in this model, would produce strands of DNA of approximately 50 kb in length. These larger DNA fragments have since been observed in every apoptotic situation in which pulsed field electrophoresis analysis has been made. However, whether the large DNA fragments serve as precursors to the internucleosomally cleaved DNA remains to be determined.

Because of its irreversibility, DNA fragmentation has been proposed as the commitment step to apoptosis [13]. It occurs early in the apoptotic process in systems such as glucocorticoid-treated rat thymocytes and late in others, suggesting that there may be cell type differences in the activation of apoptotic nucleases. Agents known to inhibit nucleolytic activity, zinc ions [14] and aurintricarboxylic acid [15], also inhibit apoptosis. If nuclease activity is blocked by other means, such as calcium chelation [16], apoptosis is also blocked. When the bacterial enzyme micrococcal nuclease is added to thymocyte nuclei, the DNA is cleaved internucleosomally, and the nuclei then undergo morphological changes similar to those observed during apoptosis [7]. This apoptotic-like response to an exogenous nuclease activity suggests that DNA cleavage is a precipitating event for apoptosis. Despite intensive study, however, the nuclease or nucleases directly responsible for the specific chromatin cleavage have not been conclusively identified. Clearly, destruction of this major cellular constituent is a key component of the apoptotic process.

Involvement of proteases

Several apoptotic events may require protease activity, one of which is the generation of cleaved chromatin. This could be achieved in two ways, the first of which would be to facilitate release of DNA from the nuclear scaffold, allowing nucleases greater access to the DNA. There are calcium-regulated serine proteases associated with the nuclear scaffold [17] whose activation cleaves the lamins A and C, resulting in disruption of the nuclear structure. This disruption could separate chromatin from its protein scaffold and allow the nuclease access to sensitive sites. A second means by which proteases could contribute to chromatin cleavage would be to activate nucleases themselves, freeing them from an inhibitory complex or conformation. The rapid death caused by the release of granzymes (serine proteases) from cytotoxic T cells and natural killer cells into the target cell includes characteristic DNA fragmentation, suggesting the released granzymes activate endogenous nucleases [18].

Activation of an endogenous nuclease

The clear demonstration of both internucleosomal and 50 kb DNA fragmentation in hormone-treated rat thymocytes [19, 20] makes these cells an excellent source for the study of nucleases involved in the apoptotic process. As mentioned previously, the nuclease or nucleases responsible for chromatin degradation during apoptosis have not yet been conclusively identified. Despite the lack of evidence for a universal apoptotic nuclease (or nucleases), it is thought that nuclease activation is common to all apoptotic pathways. DNA degradation occurs prior to the onset of morphological changes of apoptosis and is considered a commitment step to apoptosis [13]. Therefore, it is of great interest to identify the enzymes involved. There are several candidate nucleases proposed: DNAse I [21], DNAse II [22], DNAse γ [23] and NUC18 [24], as well as numerous, less-specific descriptions of calcium/magnesium-dependent nucleases [5, 25–27]. The likelihood of either DNAse I or DNAse II contributing to apoptotic DNA degradation has been questioned. For example, DNAse I has been observed in the cytoplasm, but not the nucleus, of cells [21] and has also been shown to be secreted [28]. Such characteristics seem unlikely for an apoptotic nuclease, since one would not expect the ordered and rapid process of internucleosomal cleavage to require random diffusion of the nuclease into the nucleus. Additionally, DNAse II is an acidic nuclease whose optimal pH is around 5.0 [29]. Even if an apoptotic cell were to reach a pH as low as 6.5, the enzyme would be forced to work out of its optimal niche. Again, this seems an unlikely requirement for an apoptotic enzyme.

NUC18 as a potential apoptotic nuclease

NUC18, DNAse γ and other reported nucleases, however, possess properties such as a nuclear location and pH and ion requirements similar to those expected for an apoptotic nuclease and what is actually observed in apoptotic cells (reviewed in ref. 2).

Previous studies in our laboratory identified two groups of potential apoptotic nucleases, ranging from 30 to 32 kDa and 12 to 19 kDa [30]. It may be that the DNAse γ described by Tanuma and Shiokawa [23] is the same higher molecular weight activity we reported [30]. These groups of proteins were identified using a modification of a Rosenthal and Lacks protocol [31], in which DNA is incorporated into a 15% Laemmli polyacrylamide gel [32]. Protein extracts from glucocorticoid-treated rat thymocyte nuclei are electrophoresed through these gels, which are subsequently soaked overnight and washed several times to remove the sodium dodecyl sulphate (SDS). This allows proteins to renature within the gel matrix. Calcium and magnesium ions are added at 37 °C to activate the nucleases, which digest the

DNA surrounding them in the gel. These smaller pieces of DNA diffuse out of the gel so that when the gel is stained with ethidium bromide, a small 'hole', or cleared area appears at the site where a protein has either bound to or digested the DNA. By further modifying this assay such that ^{32}P-labelled DNA was used instead, the active nuclease could be conclusively identified after drying the gel, exposing it to film and looking for cleared areas in an otherwise black autoradiograph [24]. With these modifications, an 18 kDa protein capable of degrading double stranded DNA was isolated from apoptotic rat thymocyte nuclei.

S49.1 cells treated with hormone and analysed via the radioactive gel assay displayed the 18 kD nuclease activity, as did fractions obtained after gel filtration (Biogel, P100) of steroid-treated rat thymocytes [33]. This 18 kD nuclease activity has been called NUC18 [24]. NUC18 does not cleave internucleosomally; however, apoptotic nuclear extracts that were fractionated by gel filtration possess an activity at 22.7 kD which can induce internucleosomal cleavage of substrate HeLa cell chromatin [9]. Interestingly, when control thymocyte extracts, which do not normally display cleavage activity, were fractionated by Superose 12 or a sucrose density column, internucleosomal cleavage activity at the same molecular weight was observed [9]. These data indicate that the apoptotic nuclease may exist in a latent form in normal cells and is somehow activated when the cell is induced to die. In agreement with this idea, NUC18 activity has also been observed at a low level in control nuclei [24]. NUC18 activation in lymphocytes is observed when apoptosis is induced by calcium ionophores A23187 and ionomycin [33] and protein synthesis inhibitors [34]. NUC18 is dependent on calcium and magnesium ions for activation and is inhibited in the presence of zinc ions and aurintricarboxylic acid [24]. These preliminary characterizations of NUC18 activation, taken together with its isolation from apoptotic cells, suggested that NUC18 is an apoptotic nuclease. Further analysis of NUC18 involved attempts at sequencing the protein. When two peptides resulting from tryptic digestion of NUC18 were sequenced, an almost complete homology with rat cyclophilin (Cyp) A and other members of the Cyp family was revealed [35].

Cyclophilins

Background

The sequence similarity between NUC18 and Cyp was quite surprising. Cyps were first identified as the intracellular high-affinity binding proteins for the immunosuppressor drug cyclosporin A (CsA) [36]. Cyps have been found in every tissue type and organism studied to date [37–39] and display a diverse subcellular distribution, including cytoplasmic [40–42], endoplasmic reticular [43, 42], nuclear [38, 42], mitochondrial [40], periplasmic [44–46], and even secreted [47, 48]. The ubiquitous expression of Cyps suggests they play an important role in cellular processes.

The discovery that CsA blocks the PPIase activity of Cyp spawned the theory that CsA action was based on inhibition of Cyp PPIase catalysis; i.e. the inhibition of isomerase activity prevented the proper folding of proteins that are essential to the apoptotic process [49]. However, this theory proved to have several flaws; for example, CsA analogs were tested for their ability to inhibit the immune response as well as to bind Cyps, and these two activities were shown to be separable [50]. Additionally, Cyps are expressed in a wide variety of tissues and organisms, but only a few specific tissue responses are necessary to generate immunosuppression [51]. Thus, it was reasoned that Cyp must act in another capacity with CsA to elicit the immunosuppressive response. Evidence in support of this idea was provided by Liu et al. [52], who demonstrated that a Cyp:CsA complex could bind to and inhibit calcineurin (PP2B) activity. Calcineurin plays a role in interleukin-2 expression and T-cell activation. Thus, the current theory under investigation is that CsA acts in concert with Cyp to block the signalling pathway for T-cell activation. The observation that Cyps share sequence similarity with NUC18 was unexpected and suggested a new potential role for Cyps.

Cyps as potential apoptotic nucleases

To explore further the relationship between Cyps and NUC18, antibodies directed against both proteins were prepared. Western blot analyses showed that these antibodies were cross-reactive, demonstrating a structural similarity [35]. Based on these findings, studies were performed to determine whether Cyps themselves had nuclease activity. Different sources of purified recombinant Cyps were analysed in the radioactive gel assay described previously and demonstrated an ability to degrade DNA [35]. Additionally, further studies have shown that Cyps can act as nucleases under nondenaturing conditions and can degrade a variety of nucleic acid substrates, including ssDNA and RNA [53]. Cyps have a nuclear and cytoplasmic location which could account for their ability to degrade both DNA and RNA. The characteristics of Cyp nuclease activity resemble those of apoptotic nucleases in several ways, including pH optimum, endonucleolytic activity and 3′-OH termini production in the substrate DNA. Also, Cyp addition to HeLa nuclei stimulates the production of 50 kb DNA fragments in the HeLa chromatin (unpublished observations). Taken together, these data implicate Cyp as an apoptotic nuclease, perhaps the one responsible for fragmentation of the chromatin into 50 kb lengths.

The ubiquitous expression of Cyp lends itself well to the idea of an involvement in the widespread process of

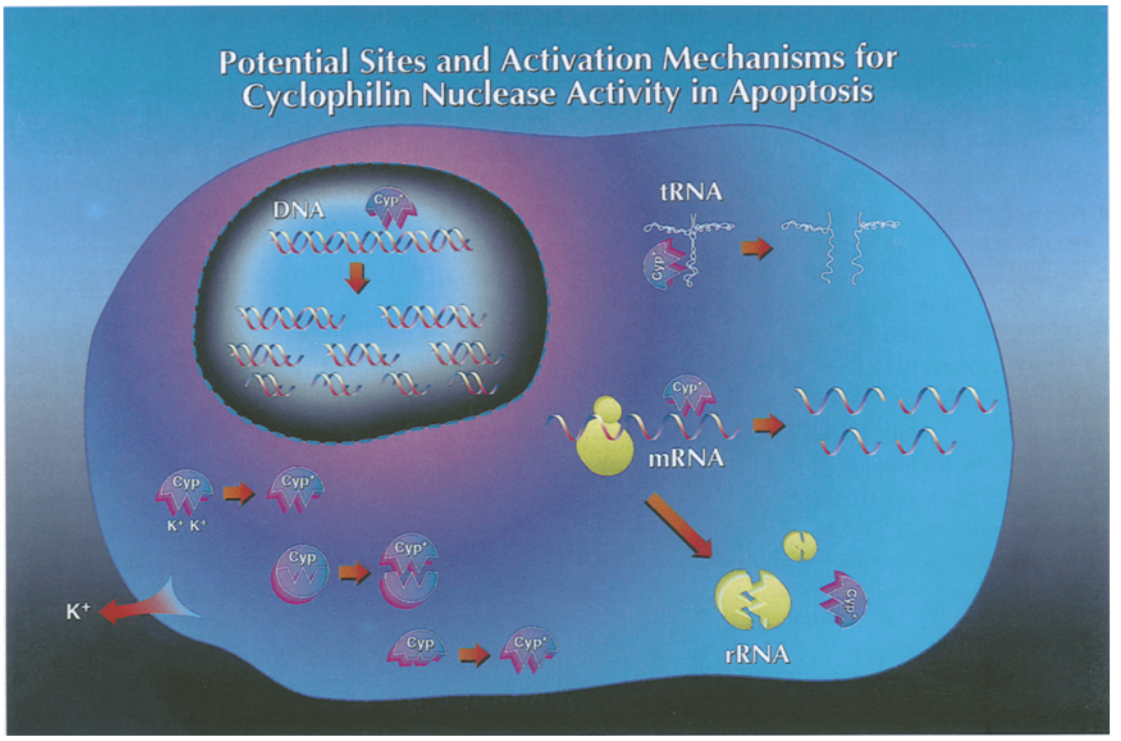

Figure 1. Potential sites and activation mechanisms for cyclophilin action in apoptosis. The different nucleic acid substrates for Cyps, both nuclear and cytoplasmic, and several possible means for activating Cyp nuclease activity are depicted in this figure. Activation of Cyp nuclease activity, represented by Cyp*, could occur through several mechanisms: the decrease in intracellular K^+ levels during the cell shrinkage process, a release of Cyp from an inhibitory complex, or a post-translational modification of Cyp. In the nucleus, Cyps could act on the chromatin to produce either 50 kb DNA fragments, or internucleosomally cleaved fragments. Our data are suggestive of Cyp producing the former pattern of DNA degradation. Cyps could be active in the cytoplasm by degrading ribosomal, messenger and transfer RNA. We have shown that different forms of Cyps, with different subcellular locations, display different nuclease characteristics. These characteristic differences may allow one Cyp to be preferentially active either in a particular subcellular compartment or against a particular nucleic acid substrate.

apoptosis. Even in single-celled organisms such as yeast, Cyps have been shown to play an essential role in death induced by certain agents. When Cyp expression is prevented in a CsA-sensitive yeast (IL993/5c), the cells no longer die in response to CsA. These Cyp knockout yeasts have no apparent abnormalities in growth or reproduction, yet their inability to die in response to CsA suggests a role for Cyp in the CsA-induced death process [53]. What remains to be determined is exactly what this role entails. The data suggest Cyps act to degrade all or some of the cell's nucleic acids. Potential sites for Cyp nuclease activity during apoptosis, as well as various activation mechanisms, are depicted in figure 1. The specificity of Cyps could be determined by either their subcellular location or by their specific type, such as Cyp A, B or C, which show differences at all levels of characterization (such as ion requirements, substrate specificity, pH requirements, products formed). A recent report describes Cyp as a DNA-binding protein, however with different ion requirements than the ones we have observed. The authors suggest that the differences are a result of investigating different forms of Cyps [54]. Indeed, even the first reports of Cyp (now called CypA) describe different isoforms [36].

The levels of Cyp proteins and the amount of Cyp nuclease activity do not change during the apoptotic process (Montague and Cidlowski, unpublished observations); therefore it is likely that Cyp nuclease activity is somehow activated during apoptosis. We propose several potential mechanisms for activation of Cyp nuclease activity. One potential mechanism is post-translational modification of Cyp. A group studying Cyp expression in differentiating keratinocytes described just this situation: a switching of levels of Cyp isoforms [56]. Because of the similarities between apoptosis and keratinocyte differentiation, this observation suggests that isoform switching may be the mode of activating Cyps during apoptosis. Another possible activation method is release of Cyps from an inhibitory complex during apoptosis. In support of this idea, previous work in our laboratory has shown NUC18 to be associated with a high molecular weight complex in control cells [24]. Cyps have also been shown to be part of a large multiprotein complex [56]. Additionally, as discussed above, CsA can enhance Cyp nuclease activity from a partially purified Cyp preparation, suggesting the binding of CsA releases Cyp from an inhibitory compound. A decrease in potassium ion levels at the onset of

apoptosis provides a third potential mechanism for activation. We have demonstrated that Cyp nuclease activity is inhibited by normal intracellular potassium levels (unpublished observations) and have observed a decrease in potassium ions during the condensation process of the cell [20]; therefore, this loss of inhibitory ions could allow Cyps to become active. Thus, there are at least three possible mechanisms to explain how Cyps could become active during apoptosis. The different subcellular localization of the Cyps could allow for simultaneous degradation of DNA and RNA, as has been observed. It will be very interesting to learn exactly what role Cyps play in the apoptotic process. The work described here has opened up the possibility that a family of proteins often overlooked as 'housekeeping' enzymes may actually prove to be vital components of a key physiological process.

In summary, we describe here a novel enzymatic activity associated with the Cyp family of proteins. These proteins display inherent nuclease activity and are capable of degrading different forms of both DNA and RNA. Characterization of this nuclease activity shows similarities with apoptotic nucleases and suggests a nucleolytic role for Cyps in apoptosis.

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