Distinct cleavage products of nuclear proteins in apoptosis and necrosis revealed by autoantibody probes

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Abstract

A central mechanism in apoptosis is the activation of proteases of the caspase (cysteine aspartases) family. Protease activation has also been implicated in necrosis, but its role in this cell death process and the identity of the proteases involved and their substrates, are unknown. Using human autoantibodies to well characterized cellular proteins as detecting probes in immunoblotting, we observed that a defined and somewhat similar set of nuclear proteins, including poly (ADP-ribose) polymerase (PARP) and DNA topoisomerase I (Topo I), were selectively cleaved during both apoptosis and necrosis of cultured cells induced by various stimuli. The resulting cleavage products were distinctively different in the two cell death pathways. In contrast to apoptosis, the cleavages of PARP and Topo I during necrosis were not blocked by the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk). These findings suggest that different proteases act in apoptosis and necrosis, and that although both cell death processes result in selective cleavage of almost identical cellular proteins, they can be distinguished immunochemically on the basis of their cleavage products.

Keywords: autoantibodies; apoptosis; necrosis; nuclear proteins; proteases

Abbreviations: caspase, cysteine aspartase; DAPI, 4',6-diamidino-2-phenylindole; $HgCl_2$, mercuric chloride; H_2O_2, hydrogen peroxide; ICE, interleukin-1β-converting enzyme; mAb, monoclonal antibody; NP-40, Nonidet P-40; OsO_4, osmium tetroxide; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; SLE, systemic lupus erythematosus; Topo I, DNA topoisomerase I; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UBF, upstream binding factor of human RNA polymerase I; U1-70 kD, 70 kD protein of the U1-small nuclear ribonucleoprotein particle; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Introduction

Apoptosis and necrosis are two morphologically distinct pathophysiological pathways of cell death (Wyllie *et al*, 1980; Buja *et al*, 1993; Majno and Joris, 1995). Apoptosis is genetically controlled and is defined by cytoplasmic and nuclear shrinkage, chromatin margination and fragmentation, and breakdown of the cell into multiple spherical bodies that retain membrane integrity. Necrosis is characterized by cytoplasmic swelling, nuclear pyknosis, and progressive loss of cytoplasmic membrane integrity (Wyllie *et al*, 1980; Buja *et al*, 1993; Majno and Joris, 1995). The latter feature leads to cellular fragmentation and release of lysosomal and granule contents into the surrounding extracellular space, with subsequent inflammation. These two cell death processes often occur simultaneously in a wide variety of pathological conditions as well as in cultured cells exposed to physiologic activators, physical trauma, or environmental toxins and chemicals (Wyllie *et al*, 1980; Arends and Wyllie, 1991; Buja *et al*, 1993; Majno and Joris, 1995; Thompson, 1995; Columbano, 1995; Rosser and Gores, 1995; Watson, 1995). The same type of insult can induce either apoptosis or necrosis but whether one mode of cell death is preferred over the other usually depends on the severity of the insult and the cell type (Lennon *et al*, 1991; Escargueil-Blanc *et al*, 1994; Bonfoco *et al*, 1995; Shimizu *et al*, 1996a). Necrosis can also occur as a secondary event following apoptosis (Majno and Joris, 1995; Tidbal *et al*, 1995). There is increasing evidence that some early biochemical events may be common to both cell death processes, while downstream mediators may be required to direct cells towards the execution of apoptosis or necrosis (Ankarkrona *et al*, 1995; Shimizu *et al*, 1996b; Leist *et al*, 1997; Leist and Nicotera, 1997).

Dissecting and discriminating the molecular mechanisms underlying apoptosis and necrosis is important for defining how cell death is regulated, understanding the role of cell death in pathology, designing approaches that could facilitate the modulation of cell death for therapeutic purposes, and establishing criteria for distinguishing unambiguously between these modes of cell death, an often difficult task, particularly in the context of pathological processes *in vivo* (Farber, 1994; Majno and Joris, 1995; Columbano, 1995). A wealth of recent evidence demonstrates that activation of a protease cascade involving members of the caspase family, formerly known as the ICE/CED-3 protease family (Alnemri *et al*, 1996), is a central mechanism in the execution of apoptosis (Nicholson, 1996). The identification of this protease family was facilitated in part by the observation that a variety of nuclear proteins, in particular PARP, were degraded in cells undergoing apoptosis (Kaufmann, 1989; Kaufmann *et al*, 1993). Many of the substrates found to be cleaved during apoptosis are protein autoantigens targeted by antinuclear autoantibodies present in the sera of patients with systemic autoimmune

diseases such as systemic lupus erythematosus (SLE), progressive systemic sclerosis (scleroderma), and Sjögren's syndrome (Casciola-Rosen *et al*, 1995; Casiano *et al*, 1996). This observation has made antinuclear autoantibodies useful probes for defining proteolytic events associated with cell death (Casiano and Tan, 1996).

There is evidence that protease activation also appears to be important in the execution of cell necrosis (Escargueil-Blanc *et al*, 1994; Shimizu *et al*, 1996b; Aguilar *et al*, 1996), but the exact role of proteolysis in this mode of cell death and the identity of the proteases involved, and their substrates, are presently unknown. Using a panel of highly specific human autoantibodies to 18 well characterized intracellular protein autoantigens as detecting probes in immunoblotting, we show here that a defined and somewhat similar set of nuclear proteins, which includes PARP and Topo I, were selectively cleaved during both apoptosis and necrosis induced by a variety of stimuli. The resulting cleavage fragments were distinctively different in the two modes of cell death. Our findings indicate that necrosis, like apoptosis, is accompanied, at least during early stages, by specific cleavage of key nuclear substrates and not by a generalized degradation of intracellular material. Moreover, the results suggest that different proteases may underlie the

execution of apoptosis and necrosis, and that these modes of cell death may be distinguished immunochemically on the basis of the cleavage products of certain nuclear proteins, in particular PARP and Topo I.

Results

### Induction of apoptosis and necrosis in Jurkat cells

Figure 1a depicts time courses showing the percentages of Jurkat cells displaying apoptotic morphology and retaining cytoplasmic membrane integrity after treatment with either anti-CD95 (Fas/APO-1) monoclonal antibody (mAb) CH-11, 10% ethanol, 0.1% hydrogen peroxide (H_2O_2) 40 μM mercuric chloride ($HgCl_2$) or heat (55°C). Cells incubated with anti-CD95 mAb maintained membrane integrity, as inferred by their ability to exclude trypan blue (Figure 1a) and their nuclei displayed the characteristic apoptotic morphology (Figure 1b). Treatment with ethanol, H_2O_2, $HgCl_2$, or heat produced a rapid decline in the number of cells maintaining membrane integrity, with no increase in the number of apoptotic cells relative to the untreated control cultures (Figure 1a), findings consistent with previous reports showing induction of cell necrosis under these conditions (Lennon *et al*, 1991; Shenker *et al*, 1993; Houge *et*
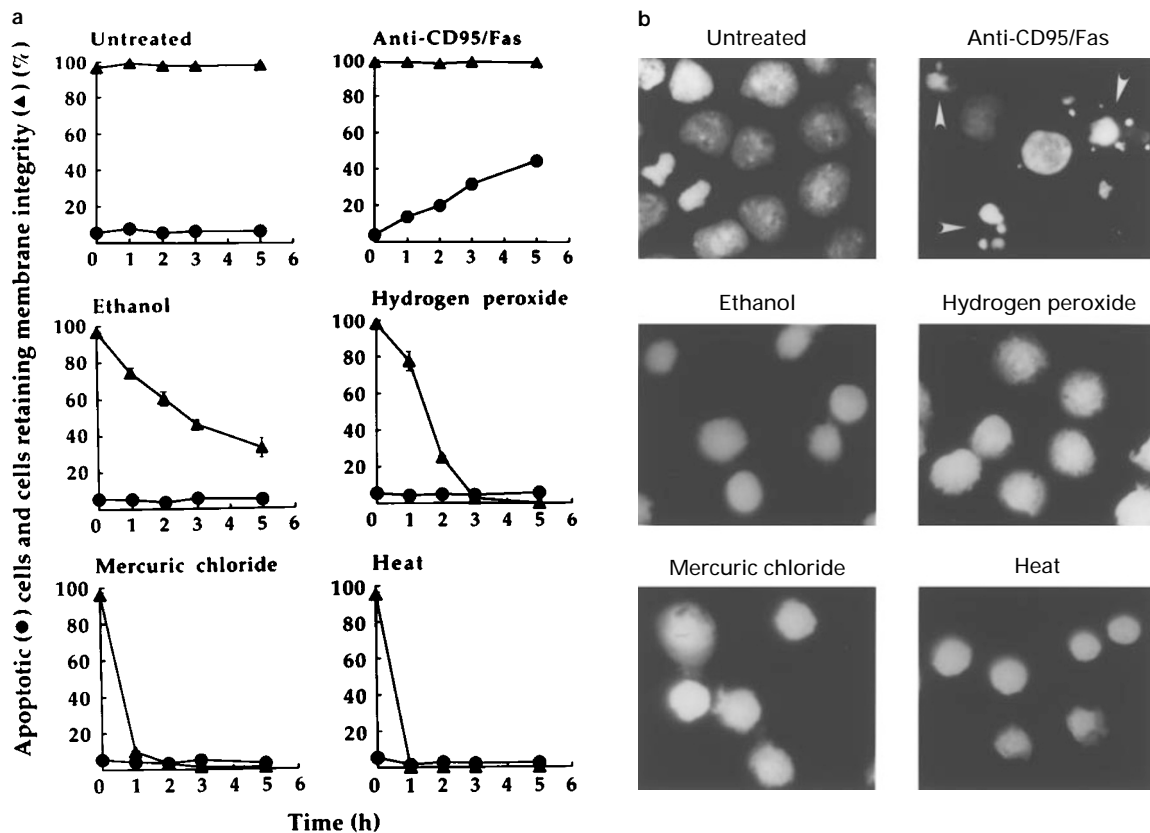
Figure 1 Induction of apoptosis and necrosis in Jurkat cells. (**a**) Progression of apoptosis and necrosis. Circles represent percentage of apoptotic cells whereas triangles represent percentage of cells retaining membrane integrity. Apoptosis was induced by exposure to anti-CD95/Fas mAb. Necrosis was induced with either 40 μM $HgCl_2$, 10% ethanol, 0.1% hydrogen peroxide, or heat (55°C). Quantitation of apoptotic and necrotic cells was performed as described in Materials and Methods. A minimum of 200 cells from different fields were evaluated in four independent experiments. (**b**) DAPI-staining of nuclei from untreated, apoptotic, and necrotic Jurkat cells. Arrowheads point to apoptotic cells showing chromatin margination and multiple apoptotic bodies containing DAPI-positive staining material

al, 1995). Treatment with HgCl$_2$ and heat produced the most dramatic effects in cell viability (Figure 1a). Nuclei from cells incubated under necrosis-inducing conditions lacked the characteristic apoptotic morphology (Figure 1b).

A more definitive morphological distinction between apoptosis and necrosis in Jurkat cells was obtained by electron microscopy (Figure 2). Untreated cells (Figure 2A) were characterized by a single large nucleus containing a prominent nucleolus. Treatment with anti-CD95 mAb (Figure 2B) led to nuclear shrinkage with the characteristic apoptosis-associated chromatin condensation and margination, and a round compact nucleolus. The cytoplasm appeared relatively normal and intact. The morphology of cells treated with ethanol, H$_2$O$_2$, HgCl$_2$, or heat was different. Ethanol treatment (Figure 2C) resulted in a shrunken nucleus with an identifiable nucleolus and condensed chromatin. Both the nucleus and the cytoplasm were extensively damaged by this treatment. H$_2$O$_2$ treatment (Figure 2D) left the nucleus and the nucleolus relatively intact, with a small amount of condensed chromatin. The cytoplasm, however, was heavily damaged, with no identifiable organelles. Treatment with HgCl$_2$ (Figure 2E) resulted in swollen and broken cells with no intact nuclei or cytoplasm. Condensed chromatin was absent but remnants of nucleoli were still identifiable. Heat treatment (Figure 2F) resulted in cells with broken nuclei and cytoplasm and condensed nucleoli.
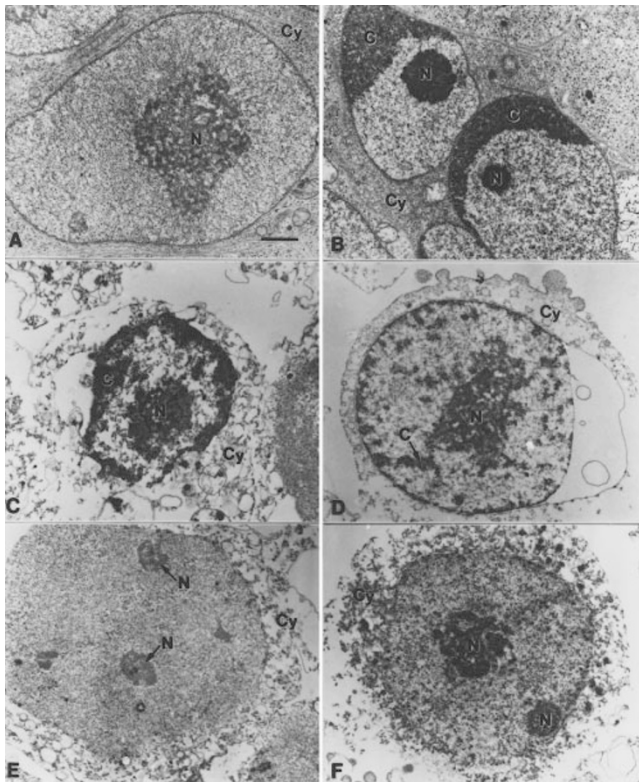
Figure 2 Electron microscopy of Jurkat cells treated for 4 h with various stimuli to induce cell death by apoptosis or necrosis. (**A**) Untreated control, (**B**) anti-CD95/Fas mAb, (**C**) 10% ethanol, (**D**) 0.1% hydrogen peroxide, (**E**) 40 μM HgCl$_2$, and (**F**) heat (55°C). N, nucleolus; C, chromatin, Cy, cytoplasm. Bar represents 1 μm

Apoptosis and necrosis of Jurkat cells are associated with selective but distinctively different patterns of nuclear protein cleavage

To determine whether necrosis is associated with cleavage of specific cellular proteins, we examined 18 well characterized protein autoantigens (nuclear and cytoplasmic) by immunoblotting of Jurkat lysates prepared from control (untreated), anti-CD95-treated, and HgCl$_2$-treated cells. Human autoimmune sera containing highly specific autoantibodies to these proteins were used as detecting reagents. Three proteins, PARP, Topo I, and the human RNA polymerase I upstream binding factor (UBF), were cleaved during both CD95-mediated apoptosis and HgCl$_2$-induced necrosis, as judged by the appearance of lower molecular weight bands in blots concomitant with a proportional reduction in the amount of the intact protein (Figure 3). Interestingly, the resulting proteolytic fragments were in general distinctively different between apoptosis and necrosis (data summarized in Table 1). The cleavage of Topo I during apoptosis generated the signature 70 kD apoptotic fragment described previously (Casiano *et al*, 1996) (Figure 3). Although this fragment also appeared during necrosis, the predominant cleavage product of Topo I in this mode of cell death was a fragment of approximately 45 kD. Other lower molecular weight cleavage products were also observed. PARP cleavage during apoptosis generated the signature 85 kD fragment (Kaufmann *et al*, 1993; Casciola-Rosen *et al*, 1995; Casiano *et al*, 1996) (Figure 3) but in HgCl$_2$-induced necrosis several bands were observed, with a predominant cleavage product of approximately 50 kD. Traces of the apoptotic 85 kD fragment were observed in some necrotic lysates. The 70 kD protein of the U1-small nuclear ribonucleoprotein particle (U1-70 kD) was cleaved during apoptosis into the signature 40 kD fragment (Casciola-Rosen *et al*, 1995; Casiano *et al*, 1996) (Figure 3). However, neither the native protein nor any cleavage fragments were detected in lysates from HgCl$_2$-treated cells. In addition, an unidentified 33 kD protein that is also recognized by this particular autoimmune serum was not detected in these lysates. Several possibilities may explain this lack of immunoreactivity, including complete protein degradation, destruction of the autoreactive epitopes by limited proteolysis, or solubilization and release of the proteins during necrosis. Consistent with our previous report (Casiano *et al*, 1996), UBF was cleaved during apoptosis into several fragments ranging from 24 kD to 55 kD (Figure 3). During necrosis, however, this protein was cleaved into fragments ranging from 45 kD to 75 kD, without detectable 32 and 24 kD fragments (Figure 3). Lamin B (68 kD) was also affected differently in apoptosis and necrosis. During apoptosis the protein was cleaved into the signature 45 kD fragment (Casiano *et al*, 1996) (Figure 3). Although a reduction in the intensity of the intact lamin B protein in necrotic lysates was observed in some blots, we were not able to detect a defined cleavage fragment with the autoimmune serum used in these experiments. This would be consistent with the recent observation that lamin B does not appear to be cleaved under conditions of ATP depletion leading to necrosis (Leist *et al*, 1997).
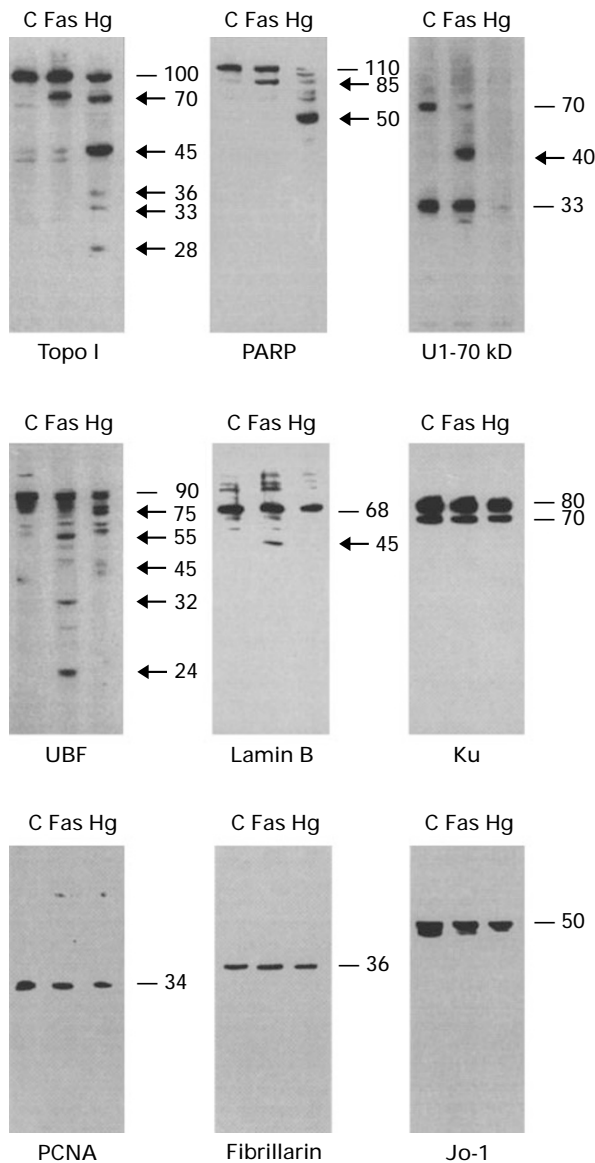
Figure 3 Cleavage of a subset of protein autoantigens during CD95/Fas-mediated apoptosis and HgCl2-mediated necrosis in Jurkat cells. Cells were exposed to either anti-CD95/Fas mAb (7 h) or 40 μM HgCl2 (5 h) prior to harvesting for lysate preparation. Representative blots of lysates from control (C), apoptotic (Fas), and necrotic (Hg) cells probed with various human autoimmune sera are shown. Intact proteins are indicated by lines, whereas proteolytic fragments are indicated by arrows. Numbers to the right of each blot represent relative molecular weight in kD

Table 1 Nuclear protein autoantigens cleaved during apoptosis and necrosis in Jurkat cells

Protein	MW	Major cleavage fragments in apoptosis	Major cleavage fragments in necrosis
Lamin B	68 kD	45 kD	Not detected
PARP	110 kD	85 kD	50 kD
Topo I	100 kD	70 kD	70, 45 kD
UBF	90 kD	multiple (24, 32, 55 kD)	multiple (45–75 kD)
U1-70 kD	70 kD	40 kD	completely degraded (?)

Cleavage fragments were detected by immunoblotting of apoptotic and necrotic cell lysates using human autoantibodies as detecting probes. Protein autoantigens that did not appear cleaved during either apoptosis or necrosis included the nuclear proteins SSA/Ro, p80 coilin, Ku, PCNA, and Sm; nucleolar proteins B23, fibrillarin, PM-Scl, and To; histidyl RNA synthetase (Jo-1); and ribosomal proteins P0, P1, and P2

A common pattern of protein cleavage is associated with different types of necrotic cell death

It was important to determine whether the protein cleavages observed during HgCl2-induced cell death could be also reproduced in Jurkat cells induced to die by necrosis with 10% ethanol, 0.1% H2O2, and 55°C heat. Figure 4a shows that under all the necrosis-inducing conditions, Topo I was predominantly cleaved into 70 and 45 kD fragments, whereas PARP was cleaved predominantly into a 50 kD fragment. An additional PARP fragment of approximately 62 kD was observed in ethanol-treated cells. The degradation patterns of UBF and U1-70 kD were also similar, respectively, under the four necrosis-inducing conditions (data not shown). To demonstrate that these necrosis-associated cleavages were not limited to Jurkat cells, necrosis was induced in HL-60 cells with 40 μM HgCl2. Immunoblotting analysis of apoptotic (induced by etoposide/VP-16) and necrotic HL-60 cells revealed patterns of protein cleavage identical to those observed in Jurkat cells (Figure 4b and data not shown). Similar results were obtained in J774 murine macrophages induced to undergo necrosis with 40 μM HgCl2 (data not shown). The observed cleavage of PARP into a 50 kD fragment during necrosis is consistent with the observation that necrosis in HL-60 cells induced by cytochalasin B is accompanied by degradation of PARP into a 50 kD polypeptide (Shah *et al*, 1996). These results suggest the activity of common proteases in different forms of necrotic cell death.

## zVAD-fmk blocks cleavages of PARP and Topo I during apoptosis but not during necrosis

We determined whether zVAD-fmk, a cell permeable tripeptide inhibitor of caspases and apoptosis (Sarin *et al*, 1996), was capable of blocking necrosis-associated proteolysis in Jurkat cells. Cells were incubated with 10 μM of zVAD-fmk for 1 h prior to induction of apoptosis with anti-CD95 mAb, or necrosis with HgCl2. zVAD-fmk blocked the morphological features of apoptosis (data not shown) as well as the cleavage of PARP and Topo I in anti-CD95 treated cells (Figure 5) but

Protein cleavage during HgCl2-induced necrosis did not appear to be indiscriminate since no evidence of cleavage was detected with our autoantibodies in many other intracellular proteins. These included the DNA binding protein Ku, PCNA, nucleolar protein fibrillarin, histidyl-tRNA synthetase (known as the Jo-1 autoantigen) (Figure 3), and the Sm autoantigen (involved in mRNA splicing), the coiled-body associated protein p80 coilin, the nuclear protein SSA/Ro, the nucleolar proteins B23, PM-Scl and To, and the ribosomal proteins P1, P2 and P0 (data not shown).

did not retard the progression of HgCl₂-induced necrosis (Table 2) nor the cleavage of PARP and Topo I during this mode of cell death (Figure 5).

Rapid cell lysis induced by non-ionic detergent results in autoantigen cleavage patterns relatively different from those generated during necrosis

To test the possibility that the autoantigen cleavages observed in necrotic cells were simply due to massive release of compartmentalized proteases during cell lysis, untreated cells were quickly lysed in buffer containing 1%

Nonidet P-40 (NP-40), 50 mM NaCl, and 10 mM HEPES, pH 7.0 (Casciola-Rosen *et al*, 1994). Lysates were incubated at 37°C for up to 5 h. Figure 6 shows that Topo I was cleaved differently in necrotic cells and in the NP-40 lysates since the 45 kD and smaller fragments generated during necrosis were not present in these lysates. Instead, proteolytic products ranging from 70–80 kD were present in the NP-40 lysates. These lysates also yielded PARP fragments of approximately 85, 62, and 50 kD (Figure 6), suggesting that proteases responsible for PARP cleavage during both apoptosis and necrosis had accessibility to this protein in these lysates. The 62 kD PARP fragment that appeared in the NP-40 lysates
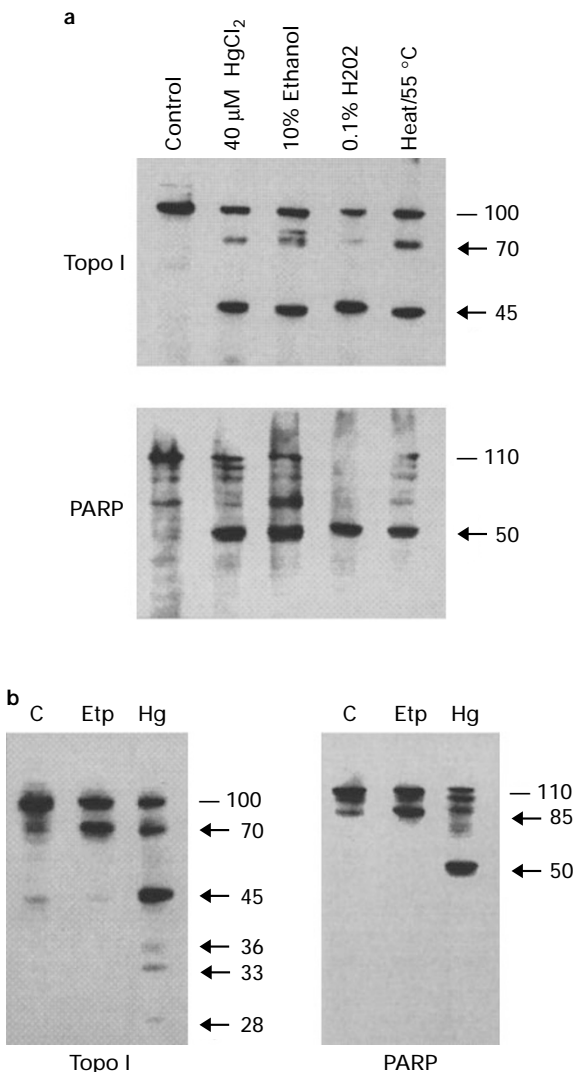
Figure 4 Immunoblots showing cleavage of Topo I and PARP in different types of necrotic cell death. (**a**) Cleavage of Topo I and PARP in Jurkat cells incubated for 5 h under various necrosis-inducing conditions. (**b**) Cleavage of Topo I and PARP during etoposide-mediated apoptosis and HgCl₂-induced necrosis in HL-60 cells. Cells were exposed to either 150 μM etoposide or 40 μM HgCl₂ for 6 h. Representative blots of lysates from control (C), apoptotic (Etp), and necrotic (Hg) cells probed with human autoimmune sera are shown. Intact proteins are indicated by lines, whereas proteolytic fragments are indicated by arrows. Numbers to the right of each blot represent relative molecular weight in kD
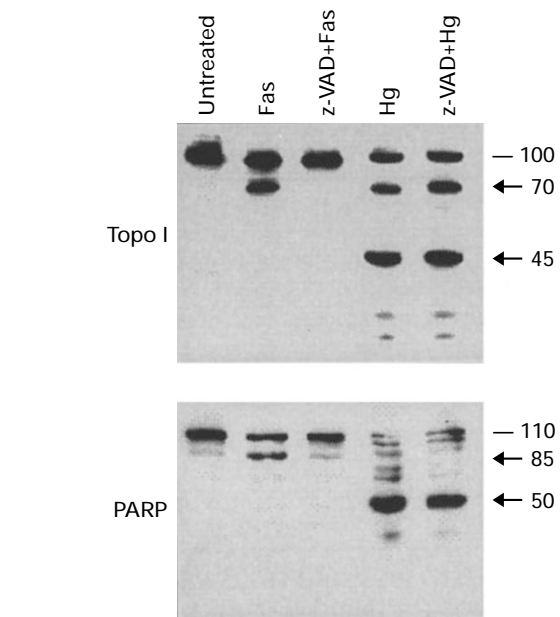

Figure 5 zVAD-fmk inhibits the proteolytic cleavages of Topo I and PARP during CD95/Fas-mediated apoptosis of Jurkat cells but has no effects on these cleavages during HgCl₂-induced necrosis. zVAD-fmk (10 μM) was added to cell cultures 1 h prior to addition of either anti-CD95/Fas mAb or HgCl₂ (40 μM). Cells were incubated for 5 h prior to harvesting for lysate preparation. Intact proteins are indicated by lines, whereas proteolytic fragments are indicated by arrows. Numbers to the right of each blot represent relative molecular weight in kD

Table 2 Assessment of loss of membrane integrity in Jurkat cells induced to die by apoptosis and necrosis after pre-treatment with zVAD-fmk

Treatment	Cell retaining membrane integrity (%)
Control	92.1±5.3
Anti-CD95/Fas mAb	90.3±5.1
zVAD-fmk+anti-CD95/Fas mAb	89.3±6.7
HgCl₂	0
zVAD-fmk+HgCl₂	0

Jurkat cells were pre-treated with zVAD-fmk (10 μM) for 1 h prior to induction of apoptosis with anti-CD95/Fas mAB or necrosis with HgCl₂ (40 μM). After incubation for 5 h, 100 μl aliquots of the cell cultures were mixed with 10 μl of a 0.4% trypan blue solution and the cells counted in a hemacytometer. At least 200 cells were counted in three separate experiments. Loss of membrane integrity was measured by the ability of cells to take up trypan blue and was considered as indicative of cell necrosis
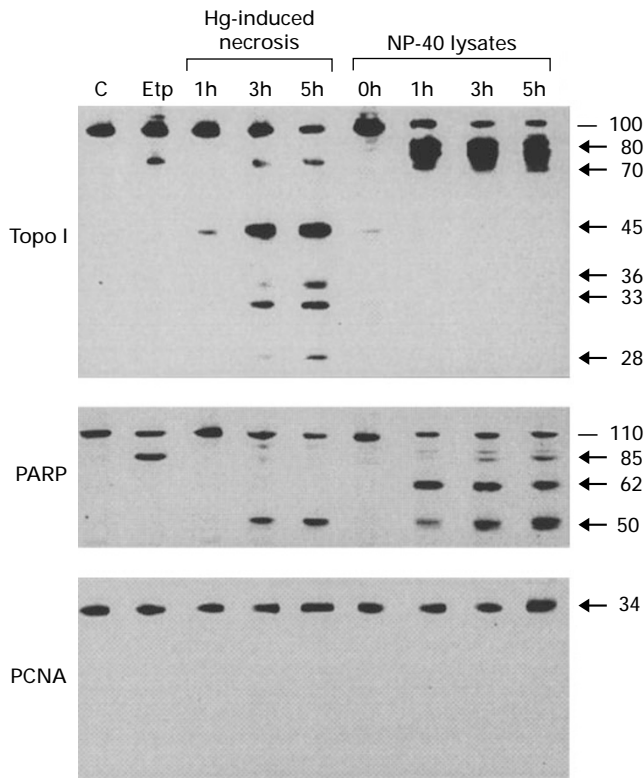
Figure 6 Proteolysis of Topo I and PARP during cell lysis induced by buffer containing NP-40. Jurkat cells were induced to die by apoptosis with 150 μM etoposide (Etp), by necrosis with 40 μM HgCl$_2$, or directly lysed in buffer containing 1% NP-40, 50 mM NaCl, and 10 mM HEPES, pH 7.0. Comparison of the autoantigen cleavage patterns in apoptotic, necrotic, and lysed cells reveals both common and different proteolytic products. Intact proteins are indicated by lines, whereas proteolytic fragments are indicated by arrows

was not observed in lysates from HgCl$_2$-treated cells, but a fragment with similar size was observed in ethanol-induced necrosis (Figure 4a). UBF, which is degraded into several fragments during necrosis, was not cleaved in the NP-40 lysates, whereas U1 – 70 kD, which is completely degraded in necrosis, also appeared completely degraded in the lysates (data not shown). Proteins that were not found cleaved during apoptosis or necrosis, such as PCNA, Ku, fibrillarin, and Jo-1 were not affected by proteases in the NP-40 lysates (Figure 6 and data not shown).

Discussion

Human antinuclear autoantibodies are valuable tools for defining proteolytic events associated with apoptotic cell death (Casciola-Rosen *et al*, 1995; Casiano *et al*, 1996; Casiano and Tan, 1996). The value of these reagents is enhanced by their reactivity with multiple epitopes within the target antigen (Tan, 1989), which facilitates the characterization of cleavage fragments that may escape detection by immunoblotting when linear sequence-targeting antibodies are used. In this study, we used antinuclear autoantibodies as probes for detecting proteolytic cleavage of certain substrates during apoptosis and necrosis. A major finding was that

necrosis is associated with proteolysis of a limited number of nuclear autoantigens, which indicates that this mode of cell death may involve, at least during the early stages, a selective rather than generalized degradation of proteins. A caveat to this finding is that proteins that appear not cleaved during cell death might be partially degraded but the degradation products are not recognized by the autoantibodies. Although not exhaustive, 18 protein autoantigens were examined by immunoblotting and, with the possible exception of lamin B, only those proteins that were degraded during apoptosis were found degraded during necrosis. In the case of U1-70 kD, failure to identify residual intact protein during necrosis might indicate extensive degradation but at the same time inability to identify cleavage fragments introduces some uncertainty to this assumption. In general, the cleavage fragments produced during necrosis were different from those produced during apoptosis, suggesting that different proteases operate in the two cell death pathways. The only cleavage fragment that was clearly common to both modes of cell death was the 70 kD polypeptide derived from Topo I, suggesting that the protease responsible for this cleavage during apoptosis might also be activated or released during necrosis. This would be consistent with recent studies showing that certain forms of necrosis can be retarded by specific inhibitors of caspases, leading to the proposal that apoptosis and necrosis may share some common mediators (Shimizu *et al*, 1996b). Alternatively, the 70 kD fragments of Topo I that arise during apoptosis and necrosis may be unrelated and generated fortuitously in both cell death processes by different proteases.

The observation that neither necrosis nor the cleavages of Topo I and PARP during this mode of cell death were blocked by zVAD-fmk implies that the caspases specifically inhibited by this tripeptide may not be involved in the execution of necrosis. This would further support the interpretation that different proteases may operate in apoptosis and necrosis. Although caspases have been clearly implicated in the cleavage of specific cellular proteins during apoptosis (Nicholson, 1996; Porter *et al*, 1997), it remains to be determined whether necrosis occurs by a pathway that is either completely or partially independent of caspase activation. It is possible that acute cell damage leading to necrosis may result in the observed proteolytic events due simply to the release of compartmentalized proteases. Our data indicate that rapid cell lysis by itself may contribute to the generation of some but not all the cleavages observed during necrosis. It cannot be ruled out the possibility that some lysosomal or cytosolic proteases which might be responsible for the necrosis-associated cleavages were inactive in our NP-40 lysates.

The present findings clearly suggest that a defined and somewhat similar set of key cellular proteins is targeted for proteolysis, albeit apparently by different proteases, regardless of how cell death is executed. The biological significance of this observation is unclear. One possibility is that these proteins may possess protease sensitive sites or motifs that are specifically targeted by proteases acting during apoptosis or necrosis. This would imply that by coincidence a similar or almost identical set of intracellular proteins possess both apoptosis-related and necrosis-

related cleavage sites. Perhaps another explanation could be that these cellular proteins are the early or key proteins targeted in either apoptotic or necrotic cell death pathways. The manner in which these proteins are cleaved may facilitate the phenotypic changes that characterize these modes of cell death. There is evidence that proteolysis of certain substrates may facilitate some of the morphological events associated with apoptosis (Rao *et al*, 1996; Liu *et al*, 1997; Porter *et al*, 1997), but similar evidence for necrosis is not yet available.

The morphological distinction between apoptosis and necrosis is relatively well defined. However, discriminating between these modes of cell death using non-morphological criteria has been more difficult due to the scarcity of biochemical or immunological markers of these cell death processes. The observed differences in the cleavage patterns of PARP and Topo I during apoptosis and necrosis should be useful for defining additional biochemical and immunological criteria which can be used to clearly discriminate between these modes of cell death. We propose that the unique cleavage products of PARP (50 kD) and Topo I (45 kD) associated with several forms of necrosis could be considered as markers of this cell death process.

Finally, analysis of protein autoantigen cleavage during apoptosis and necrosis might also provide insights into the possible role of cell death in the generation of potentially immunostimulatory forms of cellular antigens in systemic autoimmune diseases. It has been speculated that apoptotic cleavage of certain autoantigens might be a mechanism by which cryptic epitopes are revealed leading to an immunogenic response (Casciola-Rosen *et al*, 1995). In previous studies (Casciola-Rosen *et al*, 1995; Casiano *et al*, 1996) and in this study, it can be appreciated that this mechanism, if operational, would apply only to a relatively small subset of known autoantigens since the majority of autoantigens that are recognized by autoantibodies in systemic autoimmunity do not appear to be cleaved during cell death. If such an immunostimulatory mechanism exists, then both apoptosis and necrosis would need to be considered as possible processes stimulating putative autoimmune responses, since autoantigen cleavage in the two modes of cell death appears to involve a similar set of proteins.

Materials and Methods

Materials

Jurkat (human T cell leukemia) and HL60 cells (human promyelocytic leukemia) were obtained from American Type Culture Collection (Rockville, MD) and cultured under standard conditions in RPMI 1640 medium. Human autoantibodies were from the serum bank of the W.M. Keck Autoimmune Disease Center of The Scripps Research Institute. Anti-CD95 (Fas/APO-1) mAb CH-11 was from Medical and Biological Laboratories (Watertown, MA). $HgCl_2$ was from Baxter Chemical Co. (Philipsburg, NJ), H_2O_2 from Fisher (Pittsburg, PA), and ethanol from Quantum Chemical Corporation (Tuscola, IL). Etoposide was from Sigma Chemical Co. (St. Louis, MO) and zVAD-fmk from Kamiya Biomedical Company (Tukwila, WA).

Induction of cell death

Apoptosis was induced with either anti-CD95 mAb (100 – 200 ng/ml, Jurkat cells only) or by exposure of cell cultures (10^6 cells/ml) to the antitumor drug etoposide/VP-16 (150 μM), for up to 7 h. Necrosis was induced by exposure of cell cultures to conditions that have been shown in previous studies to induce necrotic cell death in a variety of cell lines (Lennon *et al*, 1991; Shenker *et al*, 1993; Houge *et al*, 1995). These conditions consisted of treatment with either 40 μM $HgCl_2$, 10% ethanol, 0.1% H_2O_2, or 55°C heat, for up to 5 h. Spontaneous cell death prior to the experiments was minimized by diluting the cell cultures in fresh medium every 2 – 3 days to maintain exponential growth.

Electron microscopy

Jurkat cells were treated for 4 h with anti-CD95 mAb to induce apoptosis and with either 40 μM $HgCl_2$, 10% ethanol, 0.1% H_2O_2, or heat (55°C) to induce necrosis. Cells were pelleted and fixed for 1 h at room temperature in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2), rinsed in cacodylate buffer, postfixed 1 h in 2% osmium tetroxide (OsO_4) buffered with cacodylate, dehydrated in a graded ethanol series, and embedded in Polybed 812 (Polysciences). Thin sections were stained with uranyl acetate and lead citrate.

Identification and quantitation of apoptotic and necrotic cells

The criteria for the identification of apoptotic cells included cell shrinkage, chromatin margination and fragmentation, and formation of apoptotic bodies, as visualized by electron microscopy as well as fluorescence microscopy of nuclei stained with 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). Cells with morphological characteristics of apoptosis excluded trypan blue. The criteria for the identification of necrotic cells included cytoplasmic swelling, nuclear pyknosis, absence of chromatin margination and fragmentation, absence of apoptotic body formation, and loss of cytoplasmic membrane integrity. Invariably, cells with morphological characteristics of necrosis were trypan blue positive, consistent with previous observations (Lennon *et al*, 1991). Quantitation of apoptotic and necrotic cells was performed essentially as described previously (Lennon *et al*, 1991). Briefly, 100 μl aliquots (10^5 cells) were removed from cell cultures, centrifuged at 2000 g for 10 min, and resuspended in 100 μl of PBS containing 2% paraformaldehyde and DAPI. The cell suspension was then applied to a glass slide and DAPI-stained nuclei were visualized by fluorescence microscopy. Cells whose nuclei unambiguously displayed the apoptotic morphology were scored as apoptotic. For the quantitation of necrotic cells, an aliquot of the cell suspension was mixed with a trypan blue solution and the positively-stained cells were counted as necrotic. At least 200 cells were counted in each experiment.

Electrophoresis and immunoblotting of cell lysates

For lysate preparation, control, apoptotic, and necrotic cells were centrifuged at 3000 r.p.m. for 20 min, followed by two washes (11 000 r.p.m. for 10 min) in PBS containing the CØMPLETE Protease Inhibitor Cocktail (Boehringer Mannheim, Germany). Cells were then resuspended at approximately 10^7 cells/ml in SDS-lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 1% mercaptoethanol, and the protease inhibitor cocktail. Prior to electrophoresis, lysates were boiled for 5 min to solubilize protein, passed through a 27-gauge needle to shear the DNA, and stored at

−70°C until required. For some experiments, control cells were lysed at a concentration of 10^7 cells/ml in buffer containing 1% NP-40, 50 mM NaCl, and 10 mM HEPES, pH 7.0. Cell lysis under these conditions occurred rapidly since visualization of cells by light microscopy minutes after addition of the lysis buffer revealed heavy cell damage with trypan blue uptake. After incubation at 37°C, these NP-40 lysates were mixed with an equal volume of SDS-lysis buffer and processed for electrophoresis as indicated above. Total protein from approximately 10^6 cells were added to individual lanes and separated by SDS − PAGE followed by transfer to nitrocellulose at 250 mA for 4 − 5 h. Immunoblotting procedures were as described previously (Casiano *et al*, 1996). Detection of bound autoantibodies was achieved using a horseradish peroxidase-conjugated goat anti-human IgG secondary antibody (Zymed, South San Francisco, CA) at a 1 : 3000 dilution, in combination with the enhanced chemilumines-cence method (ECL, Amersham Life Science, Cleveland, OH).

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