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High and low molecular weight DNA cleavage in ovarian granulosa cells: characterization and protease modulation in intact cells and in cell-free nuclear autodigestion assays

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Abstract

To continue elucidation of the biochemical and molecular pathways involved in the induction of apoptosis in granulosa cells (GC) of ovarian follicles destined for atresia, we characterized the occurrence and protease modulation of high and low molecular weight (MW) DNA fragmentation during rat GC death. Atresia of ovarian follicles, occurring either spontaneously *in vivo* or induced *in vitro*, was associated with both high MW and internucleosomal (low MW) DNA cleavage. Incubation of follicles in the presence of a putative irreversible and non-competitive inhibitor of caspase-1 (interleukin-1β-converting enzyme or ICE), sodium aurothiomalate (SAM), completely prevented internucleosomal, but not high MW, DNA cleavage. As reported previously, morphological features of apoptosis (pyknosis, cellular condensation) and atresia (granulosa cell disorganization, oocyte pseudomaturation) remained detectable in SAM-treated follicles. The potential involvement of proteases in endonuclease activation was further analyzed in cell-free assays using nuclei from both GC (which autodigest their DNA) and HeLa cells (HC, which do not autodigest their DNA unless incubated with extracts prepared from other cell types). Crude cytoplasmic extracts prepared from GC induced both high MW and internucleosomal DNA cleavage in HC nuclei. The induction of low, but not high, MW DNA cleavage in HC nuclei by GC extracts was suppressed by pretreatment of the extracts with SAM or with any one of the serine protease inhibitors, dichloroisocoumarin (DCI), N-tosyl-L-leucylchloromethylketone (TLCK) or N-tosyl-L-phenylchloromethylketone (TPCK). Interestingly, SAM and DCI also prevented cation-induced low MW DNA fragmentation in GC nuclei; however, TLCK and TPCK were without effect. Our results support a role for cytoplasmic and nuclear serine proteases in the activation of the endonuclease(s) responsible for internucleosomal DNA cleavage during apoptosis.

Keywords: apoptosis; DNA cleavage; nucleases; proteases; granulosa cell; follicle; atresia; ovary

Abbreviations: GC, granulosa cell; ICE, interleukin-1β-converting enzyme; SAM, sodium aurothiomalate; HC, HeLa cell; DCI, dichloroisocoumarin; TLCK, N-tosyl-L-leucylchloromethylketone; TPCK, N-tosyl-L-phenylchloromethylketone; IAA, iodoacetic acid; IAM, iodoacetamide; YVAD-CHO, Tyr-Val-Ala-Asp-aldehyde; DEVD-CHO, Asp-Glu-Val-Asp-aldehyde; PMSF, phenylmethylsulfonyl fluoride; PFGE, pulsed-field gel electrophoresis; CAGE, conventional agarose gel electrophoresis

Introduction

The vast majority of ovarian follicles formed during the perinatal period in vertebrate species undergo a process of degeneration referred to as atresia (reviewed in Tilly and Ratts, 1996). Recent investigations from a number of laboratories have identified the occurrence of apoptosis as an underlying feature of the atresia process in diverse species (Hughes and Gorospe, 1991; Tilly *et al*, 1991, 1992a; Tilly, 1993; Jolly *et al*, 1994; Guthrie *et al*, 1995). In most species studied thus far, apoptosis occurs predominantly in the somatic granulosa cells (GC) that support and nourish the female germ cell housed within the follicle during development. Using the rat as a model system, we and others have demonstrated that the initiation of apoptosis in GC of mature antral follicles can be modulated by gonadotrophic hormones (Tilly *et al*, 1992b, 1995a; Chun *et al*, 1994; Tilly and Tilly, 1995), locally-produced ovarian growth factors (Tilly *et al*, 1992b, 1995b; Chun *et al*, 1994; Luciano *et al*, 1994), neuropeptides (Flaws *et al*, 1995a) and cytokines (Hughes *et al*, 1994). Importantly, the actions of many of these factors appear to be linked to altered expression of several cell death-associated genes (Tilly and Tilly, 1995; Tilly *et al*, 1995a, c; Flaws *et al*, 1995b; Johnson *et al*, 1996; reviewed in Tilly, 1996 and Tilly *et al*, 1997) that have been identified as key players in apoptosis regulation in non-gonadal tissues and tumour cell lines (reviewed in Reed, 1994; Korsmeyer, 1995; Wyllie, 1995; Martin and Green, 1995; Fraser and Evan, 1996; Ko and Prives, 1996; Kumar and Lavin, 1996; Patel *et al*, 1996; Yang and Korsmeyer, 1996).

Of direct relevance to the present study, we have reported that ovarian expression of two members of the

cysteine aspartic acid-specific (*CASP*, (Alnemri *et al*, 1996); also referred to as *ced*-3/interleukin-1β-converting enzyme (*Ice*)) gene family, namely *CASP-2* (Ich-1 or Nedd-2; Kumar *et al*, 1992, 1994; Wang *et al*, 1994) and *CASP-3* (CPP32, Yama or apopain; Fernandes-Alnemri *et al*, 1994; Nicholson *et al*, 1995; Tewari *et al*, 1995), are suppressed during gonadotrophin-promoted follicular survival (Flaws *et al*, 1995b). Additionally, proteolysis of fodrin, a cytoskeletal protein and known target for caspase attack (Martin *et al*, 1995, 1996), occurs during apoptosis and atresia in mouse antral follicles incubated *in vitro* without hormonal support (Maravei *et al*, 1997), and apoptosis in this model can be blocked by treatment of follicles with specific peptide inhibitors of caspases (Maravei *et al*, 1997). Collectively, these data suggest that caspases are involved in executing the cell death command in GC during follicular atresia, consistent with the reported role of this family of proteases in mediating apoptosis in a wide variety of extragonadal cell types (reviewed in Martin and Green, 1995; Fraser and Evan, 1996; Kumar and Lavin, 1996; Patel *et al*, 1996). However, we have also observed that treatment of rat ovarian follicles in culture with a reported irreversible and non-competitive inhibitor of caspase-1 (ICE), sodium aurothiomalate (SAM; Wilson *et al*, 1994), prevents internucleosomal DNA cleavage but not morphological indexes of apoptosis in GC (Flaws *et al*, 1995b). Although the basis for the dissociation of biochemical *versus* morphological evidence of apoptosis in ovarian follicles was not investigated, we proposed that this may be due to the occurrence of high molecular weight (MW) DNA cleavage not detected by conventional agarose gel electrophoresis (CAGE).

Recent investigations using the technique of pulsed-field gel electrophoresis (PFGE) to resolve high MW DNA fractions (greater than 20 kilobases (kb)) not normally distinguishable by CAGE have suggested that sequential cleavage of genomic DNA to 300 kb rosette structures, 50 kb loops and, lastly, mono- and oligonucleosomal fragments, occurs during apoptosis (reviewed in Walker and Sikorska, 1994; Walker *et al*, 1995). Several studies have demonstrated that morphological apoptosis can in fact proceed in the complete absence of internucleosomal DNA cleavage (Cohen *et al*, 1992; Collins *et al*, 1992; Oberhammer *et al*, 1993; Zakeri *et al*, 1993; Walker *et al*, 1994); however, in these examples, rosette or loop cleavage is detectable by PFGE (Walker and Sikorska, 1994). Based on these observations, we designed the present experiments to utilize PFGE and CAGE to characterize the occurrence and protease modulation of high and low MW DNA cleavage in GC of rat ovarian follicles. We then sought to compare the data obtained from analysis of intact GC (obtained *in vivo* and from follicles incubated *in vitro* as a model for atresia) to results derived from studies of DNA cleavage using previously characterized cell-free nuclear autodigestion assays. These experiments revealed that a complex interaction likely occurs between multiple proteases and nucleases during the initiation and progression of apoptosis in GC, and that different requirements for various proteases in the apoptotic response may be related to cytoplasmic *versus* nuclear events.

Results

Analysis of DNA cleavage in GC during follicular atresia *in vivo*

The first experiments were conducted to characterize high and low MW DNA cleavage in GC during apoptosis and atresia *in vivo* using the gonadotrophin-primed immature rat model (Hughes and Gorospe, 1991; Tilly *et al*, 1992b, 1995a). By PFGE analysis, cleavage of genomic DNA into high MW fragments migrating close to the 50 kb size range (DNA loops) was detectable in GC collected from unstimulated ovaries that are known to possess a large population of atretic antral follicles (Figure 1, upper panel) (Tilly *et al*, 1995a). The occurrence of high MW DNA cleavage in unstimulated GC was mirrored by the presence of extensive internucleosomal cleavage, as determined by CAGE and autoradiography (Figure 1, lower panel). By comparison, GC obtained from healthy antral follicles of rats primed 2 days earlier with equine chorionic gonadotrophin (eCG) did not exhibit evidence of high MW DNA breakdown nor internucleosomal DNA cleavage (Figure 1, upper and lower panels, respectively), consistent with the absence of apoptosis and atresia in gonadotrophin-stimulated ovaries (Tilly *et al*, 1992b, 1995a).

High and low MW DNA fragmentation in incubated follicles: effects of SAM *in vitro*

We next determined if incubated follicles induced to undergo atresia *in vitro* by trophic hormone deprivation exhibit the same pattern of DNA fragmentation observed during atresia *in vivo*. To accomplish this, antral follicles obtained from eCG-primed ovaries were analyzed by PFGE and CAGE prior to (no apoptosis) or after a 24 h incubation without trophic hormone support (extensive apoptosis). It should be noted that the occurrence of apoptosis in GC of incubated rat antral follicles has been confirmed previously by morphological analysis (Flaws *et al*, 1995a,b). Cells within healthy follicles analyzed immediately following isolation contained only intact DNA, whereas cells within follicles incubated for 24 h exhibited both high and low MW DNA fragmentation (Figure 2). Consistent with our previous study (Flaws *et al*, 1995b), *in vitro* treatment of follicles with SAM did not prevent apoptosis, as defined by morphological criteria, in GC (data not shown). To determine if high MW DNA cleavage is associated with morphological features of apoptosis in GC exposed to SAM for 24 h, follicles were analyzed by PFGE. High MW DNA cleavage was detected in both control and SAM-treated follicles incubated for 24 h, but was absent in healthy follicles prior to culture (Figure 2, upper panel). There was no evidence of internucleosomal DNA cleavage in follicles incubated with SAM, despite the prevalence of low MW DNA fragments in follicles incubated in medium alone (Figure 2, lower panel). Thus, high MW DNA cleavage was apparently sufficient for the occurrence of morphological apoptosis in GC of SAM-treated follicles.

Analysis of protease inhibitors in HeLa cell nuclei autodigestion assays

To further assess the potential role of proteases in endonuclease activation, we switched to a cell-free assay system using isolated HeLa cell (HC) nuclei. In this assay, HC nuclei autodigest their DNA in the presence of divalent cations ($CaCl_2$ and $MgCl_2$) only when incubated with extracts prepared from other cell types (Compton and Cidlowski, 1987; Schwartzman and Cidlowski, 1991; Hughes and Cidlowski, 1997). In agreement with these previous studies, crude cytoplasmic extracts prepared from GC induced both high MW and internucleosomal DNA cleavage in HC nuclei incubated for 120 min with 5 mM each of $CaCl_2$ and $MgCl_2$ (Figures 3 and 4). Pretreatment of the GC extracts with any one of several reported protease inhibitors completely blocked (SAM; dichloroisocoumarin, DCI) or partially antagonized (N-tosyl-L-leucylchloromethylketone, TLCK; N-tosyl-L-

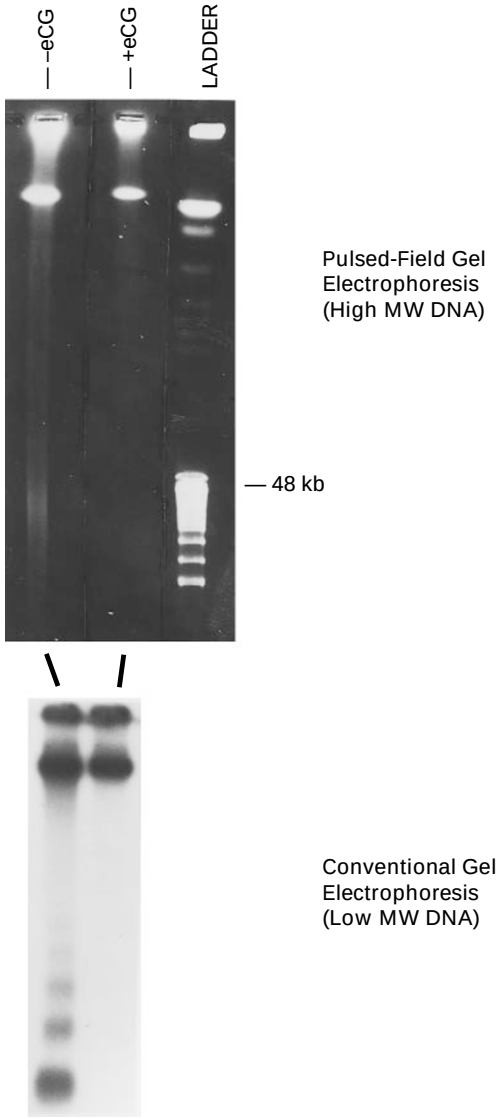

Figure 1 Analysis of high and low MW DNA cleavage in granulosa cells during atresia *in vivo*. *Upper panel*, Pulsed-field gel electrophoretic analysis of granulosa cells obtained from immature rat ovaries containing numerous atretic follicles (−eCG) and from ovaries of rats primed 2 days earlier with 10 IU of equine chorionic gonadotropin (+eCG) to suppress apoptosis and atresia *in vivo*. The migration distance of a 48 kb DNA marker fragment (LADDER) is indicated. *Lower panel*, Conventional agarose gel electrophoretic analysis of the same populations of granulosa cells described in the upper panel. Note that high and low MW DNA fragments characteristic of apoptosis were only detected in granulosa cells obtained from ovaries possessing atretic follicles (see Tilly *et al*, 1995a)

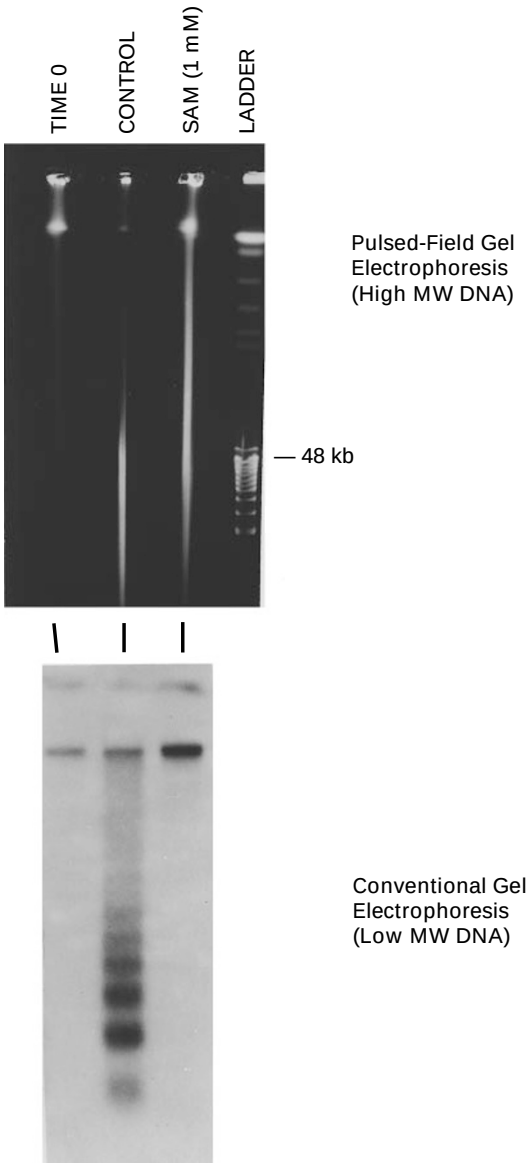

Figure 2 Analysis of high and low MW DNA cleavage in rat antral follicles incubated *in vitro*. Pulsed-field and conventional agarose gel electrophoretic analysis of DNA in rat follicles prior to incubation (TIME 0; healthy, no apoptosis) or following a 24 h serum-free incubation in the absence (CONTROL) or presence of 1 mM sodium aurothiomalate (SAM). Both control and SAM-treated follicles exhibited extensive high MW DNA cleavage (*upper panel*), contrasting the complete absence of low MW DNA fragments (*lower panel*) in follicles treated with SAM. However, SAM-treated follicles possessed widespread apoptosis in granulosa cells, as defined by morphological criteria (data not shown; see Flaws *et al*, 1995b). In the upper panel, the migration distance of a 48 kb DNA marker fragment (LADDER) is indicated

phenylchloromethylketone, TPCK) the ability of the cytoplasmic extract to induce internucleosomal DNA cleavage in HC nuclei (Figure 3, upper panel, and Figure 4). The effects of these four protease inhibitors were not mimicked by any of the eight other protease inhibitors tested (iodoacetic acid, IAA; iodoacetamide, IAM; Tyr-Val-Ala-Asp-aldehyde, YVAD-CHO; Asp-Glu-Val-Asp-aldehyde, DEVD-CHO; antipain; leupeptin; aprotinin; phenylmethylsulfonyl fluoride, PMSF) nor by the vehicles (isopropranol; dimethylsulfoxide, DMSO) used to prepare the inhibitor stocks (Figure 3, lower panel;

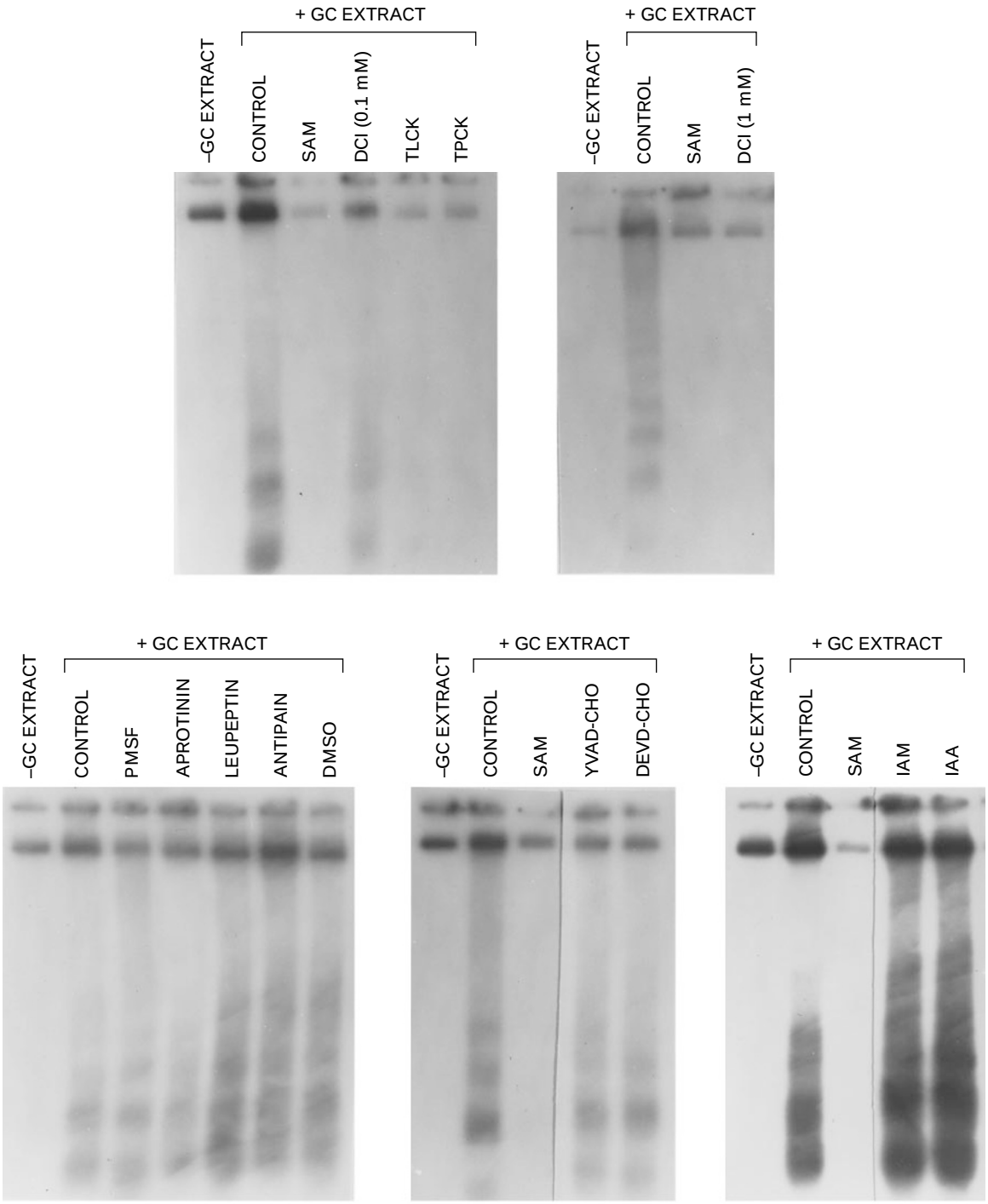

Figure 3 Qualitative analysis of low MW DNA fragmentation in HeLa cell nuclei. Nuclei isolated from HeLa cells were incubated with cations ($CaCl_2$ and $MgCl_2$, 5 mM each) in the absence (−GC EXTRACT) or presence of granulosa cell extracts (+GC EXTRACT) that had been pretreated with vehicle (CONTROL) or with one of the various protease inhibitors listed (see text for details of inhibitors and concentration used). Following incubation, DNA was extracted, quantitated, 3′-end labeled with ^{32}P-ddATP using terminal transferase (250 ng/reaction), and resolved by conventional agarose gel electrophoresis. Gels were then dried and exposed to autoradiographic films to allow visualization of internucleosomal cleavage of the DNA (see Figure 4 for quantitative assessments)

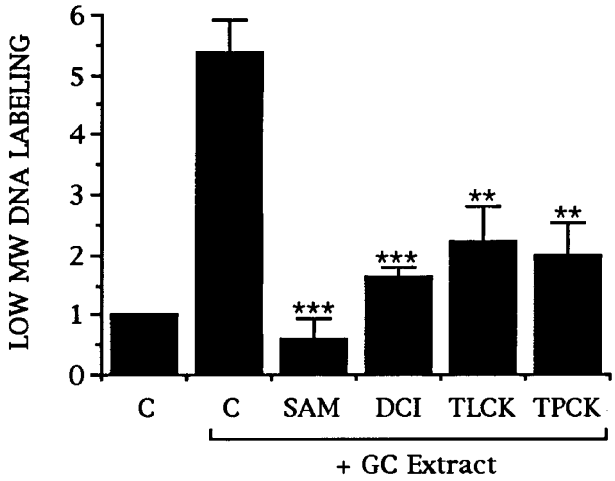

Figure 4 Quantitative analysis of low MW DNA fragmentation in HeLa cell nuclei. To provide a quantitative estimate of the effects of SAM (1 mM), DCI (1 mM), TLCK (1 mM) and TPCK (1 mM) on nuclear autodigestion using the HeLa cell nuclei assay, low MW DNA fractions (<10 kb) were excised from the gels depicted in Figure 3, mixed with scintillation fluid, and analyzed by β-counting. The extent of internucleosomal DNA cleavage is directly proportional to the number of radioactive counts per sample (for details, see Tilly and Hsueh, 1993), and the data are expressed as a fold-change *versus* control (C) without GC extracts (this data point represents the 'background' DNA cleavage in HeLa cell nuclei), which is arbitrarily set at a value of 1. The data represent the mean $\pm$ S.E.M. of results from 3–5 independent experiments for each inhibitor tested (**, $P<0.01$ *versus* no inhibitor; ***, $P<0.001$ *versus* no inhibitor)

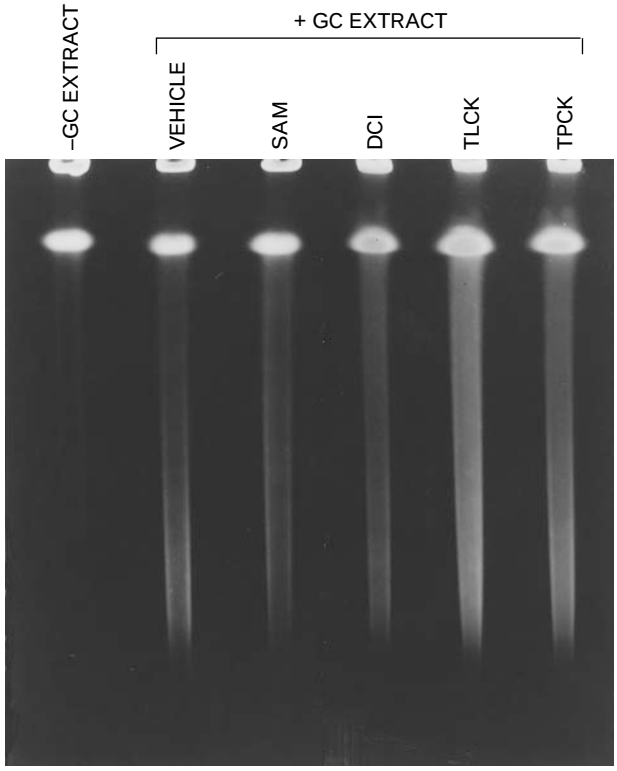

Figure 5 Pulsed-field gel electrophoretic analysis of high MW DNA cleavage in HeLa cell nuclei. Nuclei isolated from HeLa cells were incubated with cations ($CaCl_2$ and $MgCl_2$, 5 mM each) in the absence ($-$GC EXTRACT) or presence of granulosa cell extracts (+GC EXTRACT) that had been pretreated with vehicle (CONTROL) or with 1 mM of one of the following protease inhibitors: SAM, DCI, TLCK or TPCK. The nuclei were embedded in 0.5% agarose plugs and processsed for PFGE, after which the DNA was visualized by ethidium bromide staining and UV transillumination (see text for details)

quantitative data not shown). Interestingly, the occurrence of high MW DNA cleavage in HC nuclei incubated with GC extracts in the presence of divalent cations was not inhibited by SAM, DCI, TLCK or TPCK (Figure 5).

Analysis of protease inhibitors in GC nuclei autodigestion assays

Unlike HC, nuclei isolated from GC of gonadotrophin-stimulated rat ovaries exhibit rapid and extensive autodigestion of DNA following incubation with $CaCl_2$ and $MgCl_2$ (Zeleznik *et al*, 1989). To extend our investigations on the effects of GC cytoplasmic extracts on HC nuclei, we next evaluated the ability of protease inhibitors to modulate divalent cation-induced autodigestion of DNA in GC nuclei. Granulosa cell nuclei incubated in the absence of $CaCl_2$ and $MgCl_2$ exhibited little change in DNA integrity (Figure 6). However, inclusion of $CaCl_2$ and $MgCl_2$ (5 mM each) induced internucleosomal DNA cleavage (Figures 6 and 7) and caused extensive high MW DNA breakdown (Figure 8). Similar to results obtained with the HC nuclei assays, pretreatment of GC nuclei with SAM or DCI blocked the subsequent low MW DNA fragmentation elicited by addition of $CaCl_2$ and $MgCl_2$ (Figures 6 and 7); however, high MW DNA cleavage in GC nuclei was unaffected by the two inhibitors (Figure 8). In addition, no effect of the remaining 10 protease inhibitors (TLCK, TPCK, IAA, IAM, YVAD-CHO, DEVD-CHO, antipain, leupeptin, aprotinin, PMSF) or of the two vehicles (isopropanol, DMSO) on DNA autodigestion in GC nuclei was observed (Figure 6; quantitative data not shown).

Nuclease inhibitory activity of SAM

To determine if the ability of SAM or DCI to prevent DNA fragmentation in both cell-free assays (HC and GC nuclei) was related to nuclease inhibition, both compounds were tested using a cell-free nuclease activity assay that relies in the ability of nuclear protein extracts to catalyze degradation of plasmid DNA (Montague *et al*, 1997). Granulosa cell nuclear protein extracts catalyzed degradation of the linearized plasmid DNA following a 1.5 h incubation with $CaCl_2$ and $MgCl_2$ (Figure 9). Inclusion of vehicle or DCI (0.5 or 1 mM) did not affect plasmid degradation catalyzed by the GC nuclear protein extracts (Figure 9); however, SAM (1 mM) completely prevented GC nuclear protein extract-induced plasmid DNA degradation (Figure 9).

Discussion

The function of proteases, particularly the aspartate-specific cysteine proteases comprising the *CASP* gene family, in promoting apoptosis has become an area of tremendous research activity (reviewed in Martin and Green, 1995; Fraser and Evan, 1996; Kumar and Lavin, 1996; Patel *et al*, 1996). This interest stems, at least in part, from the finding that

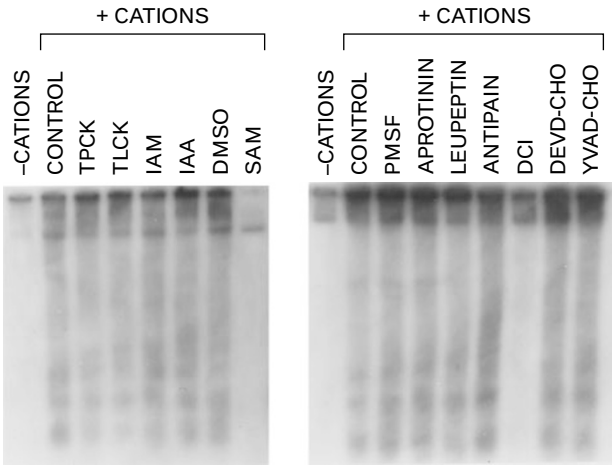

Figure 6 Qualitative analysis of low MW DNA fragmentation in granulosa cell nuclei. Biochemical analysis of DNA from granulosa cell nuclei incubated without (−CATIONS) or with (+CATIONS) $CaCl_2$ and $MgCl_2$ (5 mM each) for 30 min at 37°C following a 15 min pretreatment at room temperature in the absence (CONTROL) or presence of one of 12 different protease inhibitors (see text for details). Following incubation, DNA was extracted, quantitated, 3′-end labeled with ^{32}P-ddATP using terminal transferase (250 ng/reaction), and resolved by conventional agarose gel electrophoresis. Gels were then dried and exposed to autoradiographic films to allow visualization of internucleosomal cleavage of the DNA (see Figure 7 for quantitative assessments)

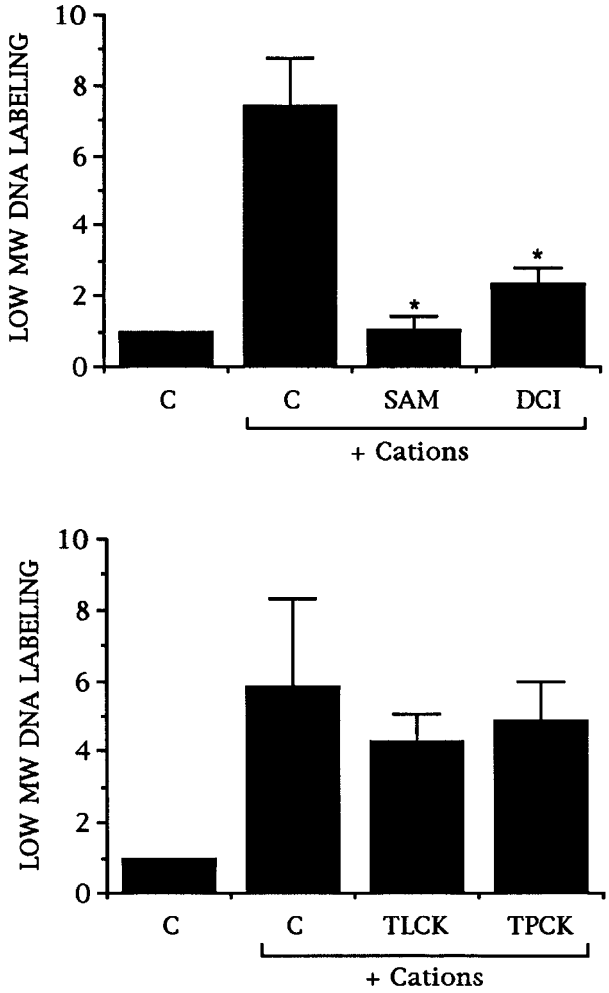

Figure 7 Quantitative analysis of low MW DNA fragmentation in granulosa cell nuclei. To provide a quantitative estimate of the effects of SAM (1 mM), DCI (1 mM), TLCK (1 mM) and TPCK (1 mM) on nuclear autodigestion using the granulosa cell nuclei assay, low MW DNA fractions (< 10 kb) were excised from the gels depicted in Figure 6, mixed with scintillation fluid, and analyzed by β-counting (see legend to Figure 4 for details). The data are expressed as a fold-change *versus* control (C) without cations (this data point represents the 'background' DNA cleavage detected in granulosa cell nuclei), which is arbitrarily set at a value of 1. The data represent the mean ± S.E.M. of results from 3–5 independent experiments for each inhibitor tested (*, $P < 0.05$ *versus* no inhibitor)

caspases represent the vertebrate counterparts to the protein encoded by the *Caenorhabditis elegans* death gene, *ced-3* (Yuan *et al*, 1993; Xue *et al*, 1996). In elegant genetic mutation analyses conducted with the nematode, functional expression of the *ced-3* gene has been demonstrated as a prerequisite event for all normal cell death to proceed during *C. elegans* development (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994). Moreover, recent data have indicated that caspases function downstream of Bcl-2-related proteins in a conserved intracellular pathway leading to apoptosis induction in diverse cells of vertebrate species (Chinnaiyan *et al*, 1996; Estoppey *et al*, 1997; Perry *et al*, 1997; Kumar, 1997), highly reminiscent of the molecular ordering of the Ced-9 death repressor protein as an upstream regulator of the Ced-3 death protease in *C. elegans* (Hengartner and Horvitz, 1994). Consequently, identification of the vertebrate Ced-3 homologs (caspases) is believed to be of crucial importance to our understanding of how apoptosis is modulated during normal tissue development and function in higher animal species. It should also be emphasized, however, that proteolytic enzymes not encoded by *CASP* gene family members, such as calpain (Squier *et al*, 1994) and as yet unidentified serine proteases (MacDonald and Cidlowski, 1981a,b; Suffys *et al*, 1988; Weaver *et al*, 1993; Chow *et al*, 1995), have also been implicated in the complex series of intracellular events leading to cell death committal.

Recently, studies of cell death in the ovary have demonstrated that apoptosis plays a fundamental role in establishment and depletion of the female germ cell reserve (Tilly, 1996; Tilly and Ratts, 1996). Furthermore, characterization of several *in vivo* and *in vitro* models for the analysis of ovarian cell demise has revealed the ovary to be extremely amenable to in-depth examination of the events leading to apoptosis in a physiologically-relevant setting of apoptosis. Using these models, we recently provided the first evidence that members of the *CASP* gene family are expressed and gonadotrophin regulated in the mammalian ovary (Flaws *et al*, 1995b), and that proteolysis of caspase substrates (i.e., fodrin) occurs during atresia (Maravei *et al*, 1997). Additionally, we reported that a putative specific inhibitor of caspase-1, SAM (Wilson *et al*, 1994), prevented internucleosomal DNA cleavage in GC of follicles undergoing atresia. However, morphological evaluations indicated wide-spread apoptosis in GC of SAM-treated follicles despite the complete absence of low MW DNA fragmentation (Flaws *et al*, 1995b). As such, it was hypothesized that SAM interfered with a critical proteolytic step in the

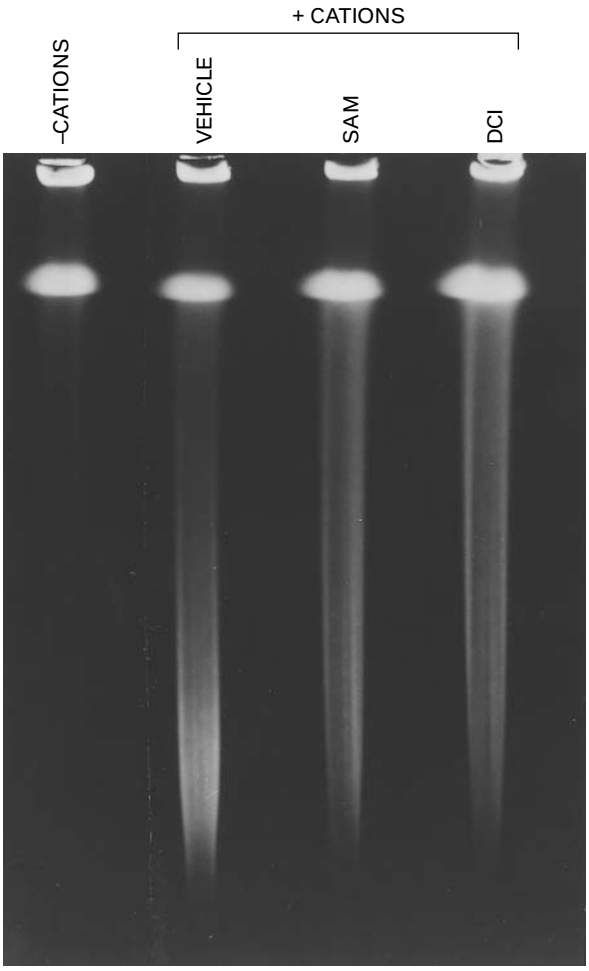

Figure 8 Pulsed-field gel electrophoretic analysis of high MW DNA cleavage in granulosa cell nuclei. Granulosa cell nuclei, incubated as described in the legend to Figure 6 without or with 1 mM SAM or DCI, were subjected to PFGE analysis. Resolved DNA was then visualized by ethidium bromide staining and UV transillumination (see text for details)

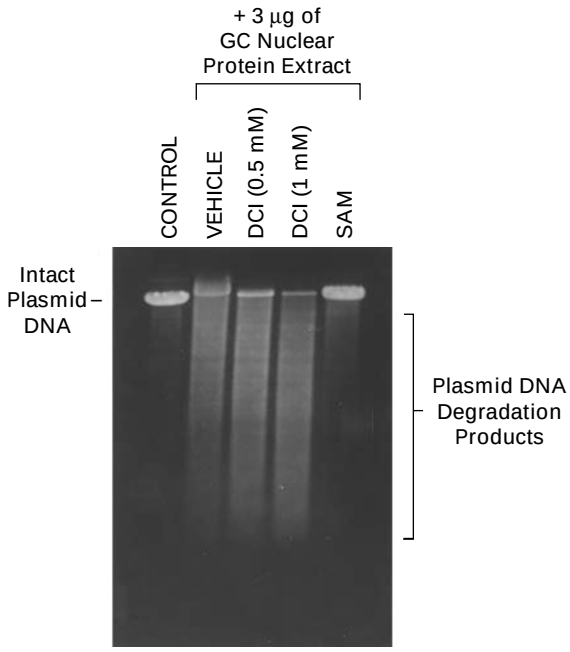

Figure 9 Analysis of nuclease inhibitory activity of SAM. Conventional gel electrophoretic analysis of pUC18 plasmid DNA integrity following incubation without (control) or with GC nuclear protein extracts in the presence of vehicle, SAM (1 mM) or DCI (0.5 or 1 mM). The plasmid was linearized with *Sma*I enzyme, mixed with 3 μg of GC nuclear protein extract in Tris-HCl/$MgCl_2$/$CaCl_2$ buffer, and incubated at 37°C for 1.5 h (see text for details). The samples were then resolved through a 1% agarose gel, and the plasmid DNA was visualized with ethidium bromide staining and UV transillumination. Note that SAM, but not DCI, prevented nuclease-mediated degradation of pUC18

apoptotic pathway leading to mono- and oligonucleosome formation in GC.

To further clarify and extend these initial investigations, herein we characterized high and low MW DNA cleavage in GC of rat ovarian follicles during atresia *in vivo* and *in vitro*. Additionally, we explored the actions of SAM, as well as several other protease inhibitors, in the activation of nucleases responsible for high and low MW DNA fragmentation in GC. Consistent with recent studies of DNA cleavage in cells of nongonadal tissues (Cohen *et al*, 1992; Collins *et al*, 1992; Zakeri *et al*, 1993; Oberhammer *et al*,. 1993; Walker and Sikorska, 1994; Walker *et al*, 1995), data from the first set of experiments confirmed that high MW DNA fragments could be detected in apoptotic GC collected from atretic follicles *in vivo*. The relative difficulty in visualizing high MW DNA cleavage during atresia *in vivo*, as compared to follicles incubated *in vitro*, probably reflects the wide heterogeneity in the extent of apoptosis in GC of follicles at varying levels of atresia. Indeed, the unstimulated immature rat ovary possesses follicles at all stages of atretic degeneration, lacking the synchrony of apoptosis induction that can be obtained by serum-free incubation of isolated follicles *in vitro* (Tilly *et al*, 1995a, 1996). Nevertheless, these data are the first to document the occurrence of high MW DNA breakdown in GC during atresia in any species, and further support the similarities in biochemical events associated with follicular atresia occurring naturally *in vivo* and induced in cultured follicles *in vitro* (Tilly *et al*, 1996).

The use of PFGE also enabled us to clarify a question raised in an earlier study using the putative inhibitor of caspase-1, SAM (Wilson *et al*, 1994), in the rat follicle culture system (Flaws *et al*, 1995b). Pulsed-field gel electrophoretic analysis of DNA within cells of follicles treated without and with SAM clearly revealed the occurrence of extensive high MW DNA cleavage in both follicle preparations, despite the complete absence of internucleosomal DNA cleavage in SAM-treated follicles. Based on these findings, and our earlier observations of the presence of apoptosis as assessed by morphological criteria in follicles treated with this inhibitor (Flaws *et al*, 1995b), we propose that high MW DNA fragmentation in the absence of internucleosomal cleavage is sufficient for apoptosis to proceed in this model system. Importantly, additional biochemical analysis of the actions of SAM, using a nuclease activity assay that minimizes any effects due to chromatin structure by employing linearized pUC18

plasmid DNA as substrate for the nuclease(s), revealed that this compound is actually a potent nuclear inhibitor. Consequently, the ability of SAM to prevent internucleosomal DNA cleavage in GC of incubated follicles may be more reflective of its actions as a nuclease, rather than a caspase-1, inhibitor. These data also indicate that extreme caution should be exercised in the interpretation of data derived from assessing the possible role of caspases in apoptosis via the use of SAM when internucleosomal DNA cleavage serves as the sole endpoint for such analyses (Kaipia *et al*, 1996).

In any case, the ability of DCI to block autodigestion of DNA into mono- and oligonucleosomal units in both the HC and GC nuclei assays reinforces the concept of protease involvement in DNA degradation during apoptosis in GC. Since DCI is characteristically described as a highly-specific serine protease inhibitor (Harper *et al*, 1985; Powers and Harper, 1986), it is likely that this, as yet unidentified, protease is not a member of the caspase family of cysteine 'death' proteases. This proposal is supported by the finding that neither of two specific and potent inhibitors of caspases, YVAD-CHO and DEVD-CHO (Thornberry *et al*, 1992; Wilson *et al*, 1994; Nicholson *et al*, 1995; Patel *et al*, 1996), were effective in modulating DNA cleavage in the GC and HC nuclei assays. The specificity of DCI action was further indicated by the relative ineffectiveness of other protease inhibitors to mimic its actions in the cell-free nuclear autodigestion experiments using GC nuclei. Included in these were the two cysteine-alkylating agents, iodoacetamide and iodoacetic acid, both of which have been reported to effectively block caspase-dependent proteolysis in various model systems (Thornberry *et al*, 1992; Miller *et al*, 1993; Casciola-Rosen *et al*, 1994; Nicholson *et al*, 1995; Xue *et al*, 1996).

The only exceptions to this finding were demonstrated by the ability of two additional serine protease inhibitors, TPCK and TLCK (Powers and Harper, 1986), to antagonize DNA cleavage in HC nuclei. Somewhat suprisingly, however, TLCK and TPCK were without effect in the GC nuclear autodigestion experiments. In previous studies by other investigators, the effects of DCI, TLCK and TPCK on various parameters associated with apoptosis, particularly at the level of DNA fragmentation, are somewhat controversial. For instance, preincubation of Jurkat cells with DCI or TPCK, but not TLCK, prevents morphological and biochemical (high and low MW DNA cleavage) aspects of apoptosis induced by activation of Fas (Weis *et al*, 1995). By comparison, both DCI and TPCK have been reported to block internucleosomal, but not high MW, cleavage of DNA in thymocytes treated with glucocorticoids (Weaver *et al*, 1993). In studies that have employed a cell-free approach similar to the one used herein, the three serine protease inhibitors found to be effective in prevention of GC cytosol-induced genome fragmentation in HC nuclei (DCI, TLCK, TPCK) were also observed at comparable doses to completely (DCI) or nearly completely (TLCK, TPCK) inhibit DNA fragmentation in HL60 nuclei exposed to cytosol prepared from HL60 cells given an apoptotic stimulus (Shimizu and Pommier, 1996). This latter study also reported that the inhibitory effects of DCI, TLCK and TPCK on DNA cleavage could not be replicated by leupeptin, iodoacetamide, YVAD-CHO nor DEVD-CHO, whereas PMSF was found to be only marginally effective (Shimizu and Pommier, 1996). Overall, the protease inhibitor profile for GC cytosol-induced DNA cleavage in HC nuclei described herein is very similar to the data obtained by Shimizu and Pommier (1996) with HL60 cell cytosol applied back to HL60 nuclei.

Although similar data were obtained, it should be pointed out that the two cell-free assays employed in our investigations were quite different than the 'homologous' cell-free approach (e.g., HL60 cytosol combined with HL60 nuclei) used by Shimizu and Pommier (1996). In the 'heterologous' HC nuclei assay, crude cytoplasmic extracts prepared from GC are believed to be the source of a protease, sensitive to inhibition by DCI, TLCK and TPCK, that is involved in catalyzing DNA digestion in HC nuclei. In contrast to this requirement for extracts from other cells to elicit DNA cleavage in HC nuclei in the presence of $CaCl_2$ and $MgCl_2$ (Compton and Cidlowski, 1987; Hughes and Cidlowski, 1997), nuclei prepared from GC of gonadotrophin-stimulated follicles are fully capable of DNA autodigestion when provided with only the two divalent cations (Zeleznik *et al*, 1989). Thus, the parallel use of these two nuclei preparations allowed us to more effectively probe the role of proteases in cytoplasmic *versus* nuclear events associated with DNA fragmentation. Indeed, our experiments indicate that two discrete pools of proteolytic activity involved in low MW DNA fragmentation may exist in GC, one present in the cytoplasm and sensitive to DCI, TLCK and TPCK, and another within the nuclear fraction sensitive to DCI only. Moreover, it is interesting to note that the extracts used for induction of DNA autodigestion in HC nuclei were prepared from a homogeneous pool of gonadotrophin-stimulated GC devoid of any apoptosis. These findings would argue that healthy GC maintain a fully functional, albeit quiescent, pathway of intracellular effectors ready to take part in the rapid execution of genome fragmentation during apoptosis. Although the identity of the signal(s) required to activate this pathway in GC remains to be elucidated, our findings suggest increased serine protease activity functions as one step in ensuring complete nuclear collapse during apoptosis.

Materials and Methods

Chemicals and reagents

Equine chorionic gonadotrophin (eCG or pregnant mare's serum gonadotrophin, PMSG; 2160 IU/mg) was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA), and the cell culture medium and supplements were from Gibco-BRL (Gaithersburg, MD, USA). Sodium aurothiomalate was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), whereas DCI, PMSF, antipain, leupeptin, aprotinin, TLCK, TPCK, IAA and IAM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The specific caspase aldehyde inhibitors, Tyr-Val-Ala-Asp-CHO (YVAD-CHO) and Asp-Glu-Val-Asp-CHO (DEVD-CHO), were obtained from Peptides International (Louisville, KY, USA).

Animals

For collection of ovarian tissues, immature (25 days) female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA), and were housed in environmentally-controlled rooms with food and water *ad libitum*. Upon arrival, rats were given a single subcutaneous injection of saline (controls), or 10 IU of eCG to promote growth of a cohort of healthy antral follicles over a subsequent 48 h period (Tilly *et al*, 1992b, 1995a). All experimental protocols involving animals were reviewed and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Additionally, all experiments conformed with procedures described in the NIH Guide for the Care and Use of Laboratory Animals.

HeLa cell cultures

For some of the cell-free nuclear autodigestion experiments, HeLa cells (ATCC CCL-2, Batch F-12477; American Type Culture Collection, Rockville, MD, USA) were cultured in Eagle's minimal essential medium (MEM) with non-essential amino acids, Earle's balanced salts solution and 10% fetal bovine serum in a humidified chamber at 37°C under 5% CO_2-95% air. Cells were harvested from the culture dishes for analysis when approximately 80% confluence was reached. Prior to collection of cells by scraping with a rubber policeman, non-adherent cells were removed by gentle washing with 2 ml of 1 × concentrated Dulbecco's phosphate-buffered saline (D-PBS). HeLa cells were selected for analysis in these experiments as previous studies have shown that HC nuclei do not exhibit autodigestion of DNA when incubated in the presence of calcium and magnesium, albeit both high and low MW DNA cleavage can be induced in the presence of these two divalent cations following incubation of HC nuclei with cellular extracts prepared from other cell types (Compton and Cidlowski, 1987; Schwartzman and Cidlowski, 1991; Hughes and Cidlowski, 1997).

Granulosa cell isolation

For analysis of high and low MW DNA integrity relative to follicular health status *in vivo*, GC were isolated by selective needle puncture of the 6–12 largest follicles present in ovaries of immature rats primed with saline (atretic follicles, apoptotic GC) or with gonadotrophin for 2 days (healthy follicles, non-apoptotic GC) as described (Tilly *et al*, 1992b, 1995a). Following estimations of cell number, aliquots of the GC were either snap-frozen for low MW DNA analysis (CAGE) or were suspended in 0.5% agarose plugs for high MW DNA analysis (PFGE). Additionally, for those experiments requiring isolation and use of GC cytoplasmic extracts and nuclei (cell-free nuclear autodigestion assays), GC were obtained by selective needle puncture of healthy antral follicles present in ovaries following 2 days of *in vivo* priming with eCG, as indicated above.

Follicle isolation and incubation

Healthy antral follicles between 700–800 μm in diameter were isolated from ovaries of rats primed for 2 days with eCG using non-enyzmatic dissection, as detailed previously (Tilly *et al*, 1992b, 1995a). Briefly, the 6–10 largest follicles in each ovary were isolated using watchmaker's forceps under a dissecting microscope, and then cleaned of adherent stromal tissue and/or small follicles. Once isolated, follicles were sized for homogeneity and either snap-frozen immediately (Time 0, no incubation) or incubated under serum-free conditions in sterile 20-ml glass scintillation vials containing 1 ml Eagle's MEM (8 follicles/culture vial) supplemented with 0.1% (w : v) bovine serum albumin (BSA, Fraction V, fatty acid free; Sigma Chemical Co.), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate for 24 h at 37°C. In some treatment experiments, follicles were incubated in the absence or presence of SAM (1 mM) for 24 h. Following incubation, follicles were collected in 12 × 75-mm polypropylene tubes, snap-frozen, and stored at −70°C until processed for analysis of low MW DNA integrity by 3′-end radiolabeling and CAGE, or were embedded in 0.5% agarose plugs for high MW DNA analysis (PFGE). For the PFGE and CAGE analyses, genomic DNA present in Time 0 follicles (no incubation) served as control data points for levels of 'background' DNA cleavage present prior to the experimental manipulations *in vitro*.

Cell-free nuclear autodigestion assays

Granulosa cells For each experimental replicate, nuclei were isolated from approximately 1.4×10^7 GC (six eCG-primed ovaries) in 1 ml of TSN buffer (10 mM Tris-HCl (pH 7.4), 25 mM NaCl, 0.34 M sucrose) by four strokes with a tight-fitting pestle in a Kontes glass homogenizer, followed by centrifugation at 800 × *g* for 10 min at 4°C. The crude cytoplasmic extract (supernatant) was collected to a 1.5 ml tube and stored on ice (for subsequent use in the HC nuclear autodigestion assay; see below), whereas the resultant nuclear pellet was gently resuspended in 1.4 ml of TSN buffer. Aliquots of the resuspended nuclei (100 μl) were dispensed into 1.5 ml tubes, and then preincubated with 1 μl of vehicle (TSN buffer, DMSO or isopropanol), SAM (1 mM final), DCI (0.1 or 1 mM final), TLCK (1 mM final), TPCK (1 mM final), YVAD-CHO (100 μM final), DEVD-CHO (100 μM final), IAA (1 mM final), IAM (1 mM final), PMSF (1 mM final), antipain (1 mM final), aprotinin (1 μg/ml final) or leupeptin (1 μg/ml final) for 15 min at 20°C. An equal volume (101 μl) of 10 mM Tris-HCl (pH 7.4) or $CaCl_2$/$MgCl_2$ (10 mM each in 10 mM Tris-HCl, pH 7.4; 5 mM each final for the autodigestion assay) was then added to the nuclei suspensions. Incubations for GC nuclear autodigestion were carried out at 37°C for 30 min (Zeleznik *et al*, 1989) after which nuclei were pelleted by centrifugation (5 min, 4°C 800 × *g*). The supernatants were removed and discarded, and the pellets were either snap-frozen for CAGE or resuspended in 50 μl of pre-warmed (37°C) 0.5% agarose for PFGE. Nuclei not incubated served as Time 0 data points for both assays.

HeLa cells For each experimental replicate, approximately 7.5×10^6 HC were homogenized in 2 ml of TSN as indicated above, and nuclei were collected by centrifugation (10 min, 4°C, 800 × *g*). Supernatants were discarded and the nuclear pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4) containing 10 mM each of $CaCl_2$ and $MgCl_2$. Aliquots (100 μl) of the HC nuclei were dispensed into 1.5 ml tubes, and then mixed with 100 μl of TSN buffer or 100 μl of GC cytoplasmic extract (equivalent to cytoplasmic extract from 10^6 cells) that had been preincubated without or with protease inhibitors (see previous section) for 15 min at 20°C. HeLa nuclei were then incubated at 37°C for 120 min (Casciola-Rosen *et al*, 1994), after which nuclei and supernatant were separated by centrifugation (5 min, 4°C, 800 × *g*). The supernatant was discarded, and the nuclear pellet was either snap-frozen for CAGE or resuspended in 50 μl of pre-warmed (37°C) 0.5% agarose for PFGE. Nuclei not incubated served as Time 0 data points for both assays. Granulosa cell cytoplasmic extract without HC nuclei served as a second control to ensure there was no nuclear DNA contribution from the homogenized GC to the HC nuclear autodigestion assay.

Pulsed-field gel electrophoresis (PFGE)

Follicles, cells or nuclei were embedded in agarose plugs (50 μl total volume, 0.5% final), and immediately immersed in 10 ml of PFGE lysis buffer (100 mM EDTA, 1% N-lauroyl-sarcosine) for overnight incubation at 37°C. Plugs were then removed, immersed in 1 ml of PFGE lysis buffer containing 50 μg/ml of proteinase-K and incubated at 50°C for an additional 12 h. Following proteolytic digestion, plugs were pre-equilibrated in 0.5 $\times$ TBE (0.89 M Tris-HCl, 0.89 M boric acid, 2.5 mM EDTA) for at least 3 h, and then subjected to PFGE using a clamped homogeneous electric field (CHEF) pulsed-field system (Bio-Rad Laboratories, Hercules, CA, USA) for 19 h at 14°C and 6 V/cm with a linear switch interval ramp from 0.5 to 45 s. These parameters were chosen to optimally separate DNA fragments ranging from 10–500 kb in a linear fashion (Birren and Lai, 1993; Hughes and Cidlowski, 1997). Size standards included chromosomes from *S. cerevisiae* and 8–48 kb DNA standards provided by Bio-Rad. Following PFGE, DNA was visualized by ethidium bromide staining and UV transillumination.

Autoradiographic analysis of internucleosomal DNA cleavage following CAGE

Genomic DNA was prepared from intact follicles or isolated nuclei as described (Gross-Bellard *et al*, 1973) and modified (Tilly and Hsueh, 1993). The quantity and purity of the DNA preparations were estimated by spectrophotometric measurements of the optical density of each sample at 260 *versus* 280 nm. Following isolation and quantitation, DNA samples (1 μg/reaction) were 3′-end labeled with [α^{32}P]-dideoxy-ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL, USA) using the terminal transferase reaction (Boehringer-Mannheim, Indianapolis, IN, USA), fractionated through conventional 2% agarose gels, and then analyzed by autoradiography and β-counting of low MW ($<$10 kb) DNA fragments, as described (Tilly and Hsueh, 1993; Tilly, 1994).

Nuclease activity assay

The nuclease activity assay employed was similar to that described for use with rat thymocytes (Montague *et al*, 1997). In brief, granulosa cells were harvested from eCG-primed rat ovaries (see 'Granulosa cell isolation' above), from which nuclei were isolated by using a Kontes Glass homogenizer (see 'Cell-free nuclear autodigestion assays' above). Nuclear extracts were prepared by freeze-thawing isolated nuclei in Tris-HCl/$CaCl_2$/$MgCl_2$ buffer followed by a 37°C incubation for 30 min. Debris and chromatin were pelleted by ultracentrifugation (100 000 $\times$ *g*) for 30 min at 4°C, and the resultant supernatant containing nuclear proteins was collected and assessed for protein content. To test for nuclease activity (see Montague *et al*, 1997), the pUC18 plasmid (Stratagene, La Jolla, CA, USA) was linearized with the *Sma*I restriction enzyme and added to tubes (1 μg/reaction) containing 3 μg of GC nuclear protein extract without or with vehicle or protease inhibitors (SAM, 1 mM; DCI, 0.5 or 1 mM) in a total volume of 10 μl (in 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$). Samples were incubated for 1.5 h at 37°C, after which 20 μg of proteinase-K (1 μl of a 20 mg/ml stock) were added followed by an additional incubation at 55°C for 1 h. Samples were then resolved through 1.0% agarose gels (1.5 h, 80 V), stained with ethidium bromide, and visualized by UV transillumination.

Data analysis and presentation

All experiments were repeated at least three times. For qualitative analysis, a representative autoradiogram or photograph (derived from results of at least three independent experiments) is presented in the Results section where appropriate. Quantitative results obtained from β-counting of low MW DNA fragments (oligonucleosomal DNA analysis) represent the mean $\pm$ S.E.M. of combined data from the replicate experiments. Statistical differences between mean values were analyzed by one-way analysis of variance followed by Scheffe's test, and significance was assigned at $P<0.05$.

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