Separate Metabolic Pathways Leading to DNA Fragmentation and Apoptotic Chromatin Condensation

By Dexter Y. Sun, Shibo Jiang,* Li-Mou Zheng, David M. Ojcius,‡ and John Ding-E Young

*From the Laboratory of Molecular Immunology and Cell Biology, The Rockefeller University, New York 10021-6399; the *Laboratory of Biochemical Virology, The New York Blood Center, New York, 10021; and ‡Unité de Biologie Moléculaire du Gène, Institut Pasteur, 75724 Paris Cedex 15, France*

Summary

Apoptosis is the predominant form of cell death observed in a variety of physiological and pathological conditions such as cancer involution, insect metamorphosis, the development of the immune and nervous systems, and embryogenesis. The typical nuclear changes taking place in apoptotic cells include extensive condensation of chromatin and internucleosomal DNA fragmentation into units of 200 base pairs. However, the mechanisms responsible for both chromatin condensation and DNA fragmentation have yet to be elucidated. In this study, micrococcal nuclease and the divalent cations, Ca^{2+} and Mg^{2+}, were applied to isolated nuclei in an attempt to reconstitute in vitro the digestion of genomic DNA associated with apoptosis. Micrococcal nuclease was found to induce a typical pattern of DNA fragmentation, but did not give rise to chromatin condensation, whereas Ca^{2+}/Mg^{2+} induced both chromatin condensation and DNA fragmentation in isolated mouse liver nuclei. When the endonuclease inhibitor $ZnCl_2$ was used, the DNA fragmentation induced by Ca^{2+}/Mg^{2+} in nuclei could be completely inhibited, but chromatin condensation still occurred. For comparison, intact liver cells were treated with valinomycin, a potassium ionophore, which gave rise to an atypical cell death, with chromatin condensation appearing without DNA fragmentation. Our results suggest that endonuclease activation in apoptosis is neither necessary nor sufficient to induce chromatin condensation, and that DNA fragmentation and chromatin condensation may be triggered through separate pathways during apoptosis.

Two types of cell death, referred to as apoptosis and necrosis, have usually been described (1–5). Apoptosis, in which the cell actively participates in its demise, is characterized by internucleosomal DNA fragmentation, chromatin condensation, and cytoplasmic blebbing. At the early stages of this process, there are no noticeable structural changes occurring within mitochondria or other cytoplasmic organelles (2, 6, 7). In contrast, necrosis differs both morphologically and biochemically from apoptosis. From the onset of necrosis, there is a marked dissolution of organized cytoplasmic structures, while the nucleus remains intact (2, 4, 8). Rather than fragmenting, the DNA breaks down at later stages into a heterogeneous mixture of fragments, visualized as a smear on gels, indicating nonspecific decomposition of the DNA. Apoptosis requires that the dying cell be metabolically active, and the process of apoptosis is often dependent on RNA and protein synthesis (9, 10). Necrosis, on the other hand, is a passive process that proceeds independently of the metabolic state of the cell.

Cell death by apoptosis is an essential feature of many normal processes and pathological conditions (11, 12). For instance, extensive apoptotic cell death occurs during embryonic development (13–15), during the hormone-regulated involution of tissues (16, 17), in immune cell selection and immunologic response (18, 19), and in aging (20). It can also be induced in various experimental systems, such as thymocytes treated with glucocorticoids (21), resting lymphocytes following low levels of γ-irradiation (22), targets of T cell killing (23, 24), tumor cells and thymocytes exposed to extracellular ATP (8), as well as tumor cell lines treated with potassium ionophores and immature murine thymocytes treated with calcium ionophore (25, 26), growth factor–dependent cells upon removal of the growth factor (27, 28), tumor regression induced with APO-1 antibody (29), and upregulation of the c-*myc* oncogene in fibroblasts (30).

DNA fragmentation is a precocious event in glucocorticoid-induced apoptosis in thymocytes, and it has been proposed that the DNA cleavage may be due to activation of a $Ca^{2+}/$

Mg^{2+}–dependent endonuclease (9, 21, 31). A number of attempts have been made to isolate this endonuclease. Preliminary data suggested that the mediator responsible for the genomic digestion during apoptosis may be a DNase I–like endonuclease (32, 33). In most cases of apoptosis, DNA fragmentation induced by endonuclease cleavage has also been associated with chromatin condensation (6, 34). In fact, Arends et al. (31) have proposed that both chromatin condensation and DNA fragmentation may be the direct result of nuclease activation within the dying cells. However, other researchers (35, 36) have advanced evidence that chromatin condensation may be triggered through endonuclease-independent pathways.

Several cytosolic and membrane bound factors, including components of the signal transduction pathway, are thought to mediate the outside signals that trigger apoptosis (37). To simplify the analysis of the metabolic events taking place at the nuclear level, we used isolated nuclei to mimic the nuclear changes associated with apoptosis. Specifically, we have used this simple in vitro system to address the question of whether endonuclease activation is in fact responsible for both DNA fragmentation and chromatin condensation. Our findings indicate that selective activation of an endonuclease is required for DNA fragmentation, but it is neither necessary nor sufficient to induce nuclear chromatin condensation. In addition, we demonstrate that DNA fragmentation and the morphological nuclear changes associated with apoptosis could be initiated within the dying cell by different mediators.

Materials and Methods

Cells and Materials. The murine tumor cell lines, P815 (mastocytoma), Yac-1 (lymphoma), and EL4 (thymoma) were maintained as suspension cultures in αMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum. Cells were harvested during log phase growth for isolation of nuclei. Livers were aseptically removed from Swiss mice (Charles River Laboratories, Wilmington, MA) and homogenized in a buffer containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 250 mM sucrose. Single cell suspensions were prepared by mincing the liver with the hub of a plastic syringe and passing the cells through a steel mesh into αMEM.

Isolation of Nuclei. Nuclei were isolated through the method described by Arends et al. (31), with minor modifications. Cells were suspended in 20 ml isotonic lysis buffer containing 100 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, supplemented with 0.15% NP-40 and 1 mM PMSF. After incubation on ice for 30 min, the lysate was centrifuged at 200 *g* to pellet the nuclei. The nuclei were washed once with 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and resuspended in a final volume of 1 ml of the same buffer.

Treatment of Isolated Nuclei with Different Agents. Nuclei were used at a concentration of 1–2 × 10^7/ml in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. Treatment of isolated nuclei with micrococcal nuclease was performed by the method of Arends et al. (31) with minor modifications. The nuclei were incubated with 0.25 μg/ml micrococcal nuclease at 37°C for varying times (Worthington Biochemical Corp., Freehold, NJ), or for 10 min with micrococcal nuclease at various concentrations, as described in results. For activation of endogenous endonucleases, nuclei were incubated in $CaCl_2$ and $MgCl_2$ at various concentrations for 3–4 h at 25°C or varying times in 1.2 mM $CaCl_2$ and $MgCl_2$, following the method of Cohen and Duke (9) with minor modifications. For inhibition of endogenous endonucleases, liver nuclei were preincubated with 100 μM or 150 μM $ZnCl_2$ for 1 h followed by incubation in 0.9 mM or 1.2 mM $CaCl_2$ and $MgCl_2$ at 25°C for 3–4 h. $ZnCl_2$ concentrations and the inhibition period were adopted from the method of Cohen and Duke (9). After treatment, nuclei were subjected to electron microscopy or used for the DNA assay.

To establish the kinetics of DNA digestion, nuclei at a concentration of 2 × 10^6/ml were incubated in 0.25 μg/ml micrococcal nuclease at 37°C. The digestion was terminated between 1 and 10 min by the addition of EDTA at a final concentration of 5 mM. Alternatively, nuclei were incubated for 10 min at 37°C with 1.2 ng/ml–1.25 μg/ml micrococcal nuclease. Mouse liver nuclei were subjected to various concentrations of $CaCl_2$ and $MgCl_2$ between 0.075 and 4.8 mM at 25°C for 3 h or to 1.2 mM $CaCl_2$ and $MgCl_2$ at different time points between 0 and 5.5 h.

Treatment of Mouse Liver Cells with Valinomycin. Mouse liver tissue was cut into very small pieces and incubated in αMEM culture medium for 4 h at 37°C in the presence or absence of 100 μM valinomycin. After incubation, liver cells were fixed in 4% glutaraldehyde, 1% paraformaldehyde, 100 mM phosphate, pH 7.2, and prepared for electron microscopy. DNA was extracted from liver cells after incubation with 100 μM valinomycin for 4, 12, or 24 h.

DNA Fragmentation Assays. For DNA gels, 2 × 10^7 nuclei were lysed by overnight incubation at 37°C in 2× DNA extraction buffer (20 mM Tris-HCl, 20 mM EDTA, 300 mM NaCl, 1.0% SDS, 200 μg/ml proteinase K, pH 8.0) with gentle rotation after treatment with micrococcal nuclease, $CaCl_2$, $MgCl_2$, or $ZnCl_2$. The DNA was extracted with phenol and chloroform three times. DNA was precipitated from the aqueous phase for 30 min on dry ice, after the addition of 0.1 vol of 3 M Na_2 acetate and 2.5 vol of 100% ethanol. The DNA was recovered by centrifugation at 13,000 *g* for 30 min. The DNA pellet was resuspended in TE (10 mM Tris, 1 mM EDTA) and digested with RNase A (50 μg/ml) at 37°C for 30 min. The same amount of nucleic acid from each sample (10–30 μg) was subjected to electrophoresis on a 1.2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

For the kinetic studies of genomic digestion, at the end of the incubation period the nuclei were harvested by centrifugation at 200 *g* for 10 min. The supernatant was saved and the pellet was lysed by adding 0.5 ml TTE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, and 0.2% Triton X-100) and vigorously vortexing at 25°C for 20 min. Fragmented DNA was separated from intact chromatin by centrifugation at 13,000 *g* for 20 min at 4°C (21). The supernatants were decanted and saved; the pellets were resuspended in 0.5 ml of the same solution. The DNA was precipitated with 25% TCA at 4°C overnight. Pellet and supernatant fractions were assayed for DNA content by the diphenylamine reaction (38) and were quantified spectrophotometrically at 630 nm using a microplate reader (model MR 700; Dynatech Laboratories Inc., Alexandria, Virginia).

Electron Microscopy. Nuclei were fixed in situ by mixing with an equal volume of fixative (4% glutaraldehyde, 1% paraformaldehyde, and 100 mM phosphate, pH 7.2) at 4°C overnight. After two washes in 200 mM phosphate, the nuclear pellets were washed and dehydrated in 30, 50, 70, 95, and 100% ethanol, and 100% propylene oxide, two times each, for 5 min. The samples were embedded in Epon at 37°C overnight and at 60°C for another 3 d. Ultrafine sections were cut on an ultramicrotome (model MT600-

XL; Reichert Scientific Instruments, Buffalo, NY). The sections were contrasted with uranyl acetate and lead citrate for examination on an electron microscope (model 100 EX; Jeol Ltd., Tokyo, Japan).

Results

Micrococcal Nuclease Induces DNA Fragmentation without Chromatin Condensation in Isolated Nuclei. It has been shown previously that the DNA laddering pattern induced during apoptosis by endonuclease activation can be mimicked by the addition of micrococcal nuclease to isolated thymocyte nuclei (31). We isolated nuclei from the cell lines EL4, P815, Yac-1, and mouse liver cells to reproduce this pattern of micrococcal nuclease-induced DNA fragmentation. These nuclei are ideal for studies on chromatin condensation, since they exhibit homogeneous chromatin structure consisting largely of euchromatin. This experiment was designed to determine whether digestion of genomic DNA is sufficient or even necessary for chromatin condensation to occur. Nuclei were subjected to digestion with 0.25 μg/ml micrococcal nuclease at 37°C between 1 and 10 min or digested at varying concentrations between 1.2 ng/ml and 1.25 μg/ml for 10 min. After digestion with 0.25 μg/ml micrococcal nuclease for 5 or 10 min, a DNA ladder pattern, similar to that described for apoptosis, was observed (Fig. 1). In contrast, untreated nuclei retained the intact high molecular weight DNA. Morphologically, in nuclei treated with 0.25 μg/ml micrococcal nuclease at different time points between 1 and 10 min, no chromatin condensation was observed, although the nuclei appeared progressively bare with increasing digestion times, presumably due to loss of DNA by digestion (Fig. 2, *b–c*). After digestion with micrococcal nuclease, a thin layer of undigested heterochromatin, not corresponding to chromatin condensation, appeared around the periphery of the nuclei in P815 (Fig. 2, *e–f*), Yac-1, and EL4 nuclei (data not shown). As expected, the chromatin in untreated nuclei was found to be homogeneously distributed and showed no condensation (Fig. 2, *a* and *d*).

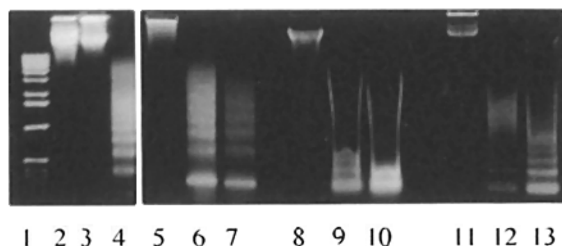

Figure 1. DNA fragmentation in isolated nuclei due to micrococcal nuclease treatment. (Lane *1*) 1-kb DNA ladder marker from GIBCO BRL, containing the following fragments: 12,216, 5,090, 4,072, 3,054, 2,036, 1,636, 1,018, 506, 396, and 344 bp each. (Lane *2*) Mouse liver nuclei incubated with 50 mM Tris-HCl, pH 7.5, for 10 min. (Lane *3*) Mouse liver nuclei incubated with 125 mM KCl, 2 mM K_2PO_4, 25 mM Hepes and 4 mM $MgCl_2$, pH 7.0, for 10 min. (Lane *4*) Mouse liver nuclei treated with 0.25 μg/ml micrococcal nuclease for 10 min. (Lanes *5*, *8*, and *11*) Contain DNA from untreated EL4, P815, and Yac-1 nuclei, respectively. DNA fragmentation was observed after treatment with 0.25 μg/ml micrococcal nuclease for 5 and 10 min in EL4 nuclei (lanes *6* and *7*, respectively), P815 nuclei (lanes *9* and *10*), and Yac-1 nuclei (lanes *12* and *13*).

The effects of different concentrations of micrococcal nuclease and different digestion times on DNA degradation were next assessed, in order to determine if there is a correlation between the nuclear morphology and DNA fragmentation under a range of different conditions. DNA fragmentation in nuclei treated with 0.25 μg/ml micrococcal nuclease could be observed within 10 min (Fig. 3 *a*), which resulted in about three quarters of the DNA in P815, Yac-1 and mouse liver nuclei being fragmented. DNA fragmentation induced by micrococcal nuclease during a 10-min digestion was also dose dependent, as shown by the diphenylamine reaction (Fig. 3 *b*). Addition of 1.25 μg/ml micrococcal nuclease for 10 min induced 90% DNA fragmentation in P815, 87% in Yac-1, and 79% in mouse liver nuclei. Again, there was no chromatin condensation with any concentration of micrococcal nuclease used (data not shown). In these experiments, the spontaneous DNA fragmentation measured by the diphenylamine reaction was <17% after a 15-min incubation in the absence of nuclease; this level may be due to mechanical damage incurred by the nuclei during the isolation procedure. However, the nuclei with spontaneously fragmented DNA displayed no chromatin condensation.

The fact that DNA fragmentation could be induced in the absence of chromatin condensation at different time points and concentrations in all four types of nuclei indicates that the nuclear changes induced by micrococcal nuclease are not limited to a single cell type.

$CaCl_2$ and $MgCl_2$ Induce both DNA Fragmentation and Chromatin Condensation in Isolated Mouse Liver Nuclei. In thymocytes, rat liver cells, and spleen cells, it has been shown that there is a Ca^{2+}/Mg^{2+}–dependent endonuclease that may cause DNA fragmentation (9, 39). To compare the nuclear changes induced by micrococcal nuclease with those resulting from activation of an endogenous Ca^{2+}/Mg^{2+}–dependent nuclease, isolated nuclei from EL4, Yac-1, P815, and mouse liver cells were incubated in the presence of Ca^{2+}/Mg^{2+}. DNA laddering was only detected in treated mouse liver nuclei, suggesting that only mouse liver nuclei among the nuclei tested contain an endonuclease whose activity can be directly activated by Ca^{2+}/Mg^{2+}; thus only these liver nuclei were used in the subsequent experiments. Incubation with 0.9 mM $CaCl_2$ and $MgCl_2$ at 25°C for 1.5 or 3 h resulted in a typical DNA ladder pattern, whereas untreated nuclei retained an intact high molecular weight DNA band upon electrophoresis through a 1.2% agarose gel (Fig. 4). Furthermore, the internucleosomal DNA fragmentation triggered by $CaCl_2$ and $MgCl_2$ was both dose and time dependent. Addition of 4.8 mM $CaCl_2$ and $MgCl_2$ induced 82% DNA fragmentation after a 3-h incubation (Fig. 5 *a*). After 5.5 h, 1.2 mM $CaCl_2$ and $MgCl_2$ induced 77% DNA fragmentation (Fig. 5 *b*).

The nuclear morphology was next monitored by electron microscopy. In contrast to the results obtained using micrococcal nuclease digestion, the nuclei treated with 0.9 mM $CaCl_2$ and $MgCl_2$ for 3 h had a typical pattern of chromatin

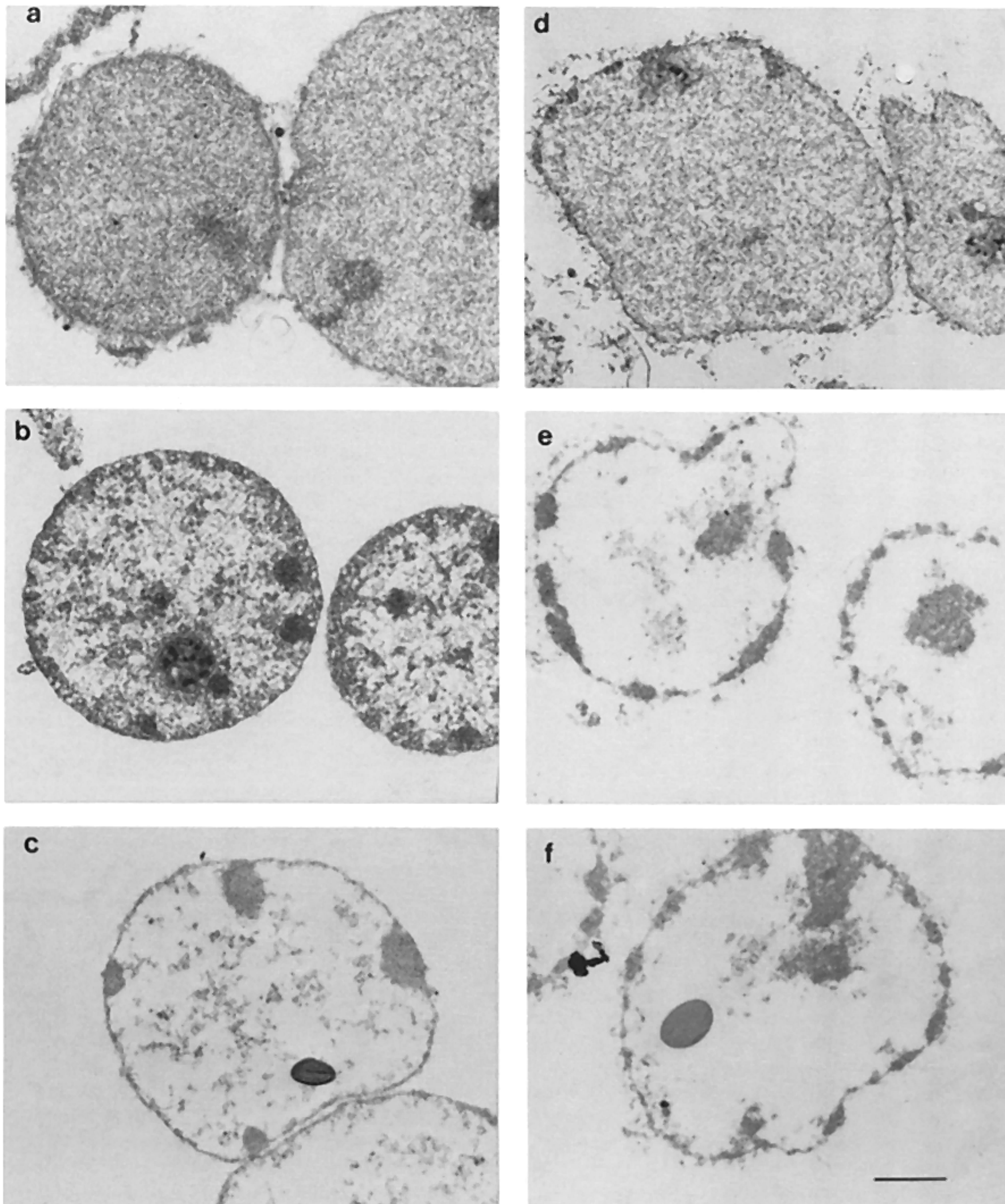

Figure 2. Nuclear changes in isolated nuclei induced by micrococcal nuclease. (*a*) Untreated mouse liver nuclei contain homogeneous nuclei matrix and euchromatin. On the periphery, there is a thin layer of condensed heterochromatin. (*b*) Mouse liver nuclei incubated with 0.25 μg/ml micrococcal nuclease for 5 min; (*c*) after a 10-min incubation, the nuclei become flocculent. There is no chromatin condensation after micrococcal nuclease digestion for either 5 or 10 min (*b* and *c*). (*d*) Untreated P815 nuclei contain nuclei with a homogeneous appearance. (*e*) There is considerable thinning of the P815 nuclei after they are digested with 0.25 μg/ml micrococcal nuclease for (*e*) 5 or (*f*) 10 min. Bar, 2 μm.

condensation (Fig. 6 *b*). A more striking level of chromatin condensation was found after treatment with 1.2 mM $CaCl_2$ and $MgCl_2$ for the same period of time (Fig. 6 *c*).

$ZnCl_2$ Inhibits the DNA Fragmentation Induced by $CaCl_2$ and $MgCl_2$, but Is Unable to Block the Chromatin Condensation in Mouse Liver Nuclei. Since $ZnCl_2$ has been shown to inhibit endogenous endonucleases in several systems (9), it is possible to establish whether endonucleases are necessary for both the DNA fragmentation and chromatin condensation caused by Ca^{2+}/Mg^{2+} or only for the DNA fragmentation. Thus, mouse liver nuclei were incubated with 100 or 150 μM $ZnCl_2$ at 37°C for 30 min and subsequently treated

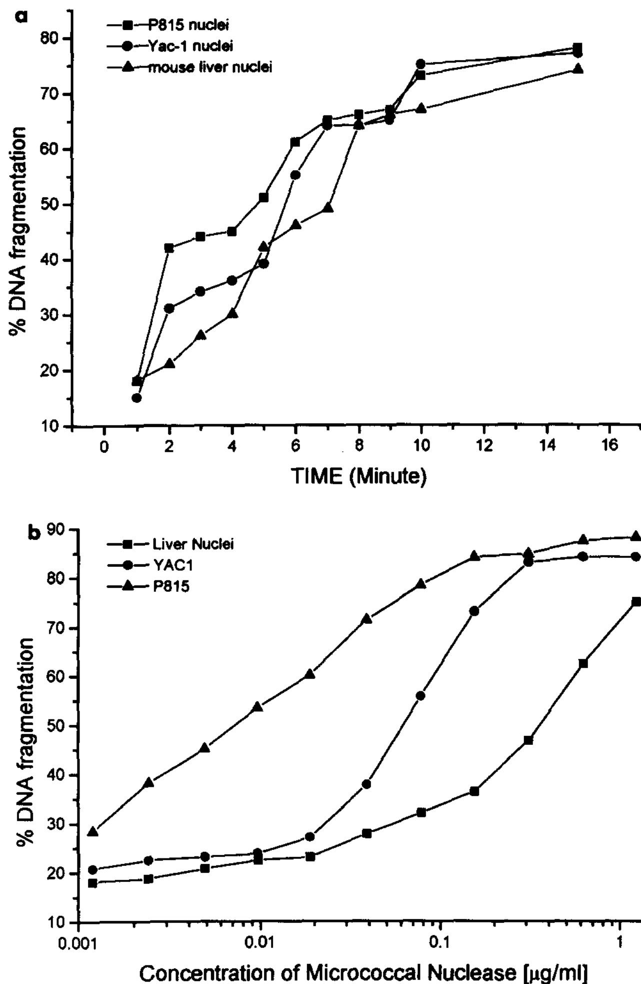

Figure 3. Kinetics and concentration dependence of DNA digestion by micrococcal nuclease in isolated nuclei, as measured by the DNA fragmentation assay. (*a*) Time dependence of DNA digestion. The DNA fragmentation in P815 and Yac-1 nuclei and mouse liver nuclei incubated with 0.25 µg/ml micrococcal nuclease increased monotonically for 10 min, before reaching a plateau. (*b*) Dose dependence of DNA digestion induced by micrococcal nuclease at concentrations between 1.2 ng/ml and 1.25 µg/ml for 10 min. After a 15-min incubation in the absence of nuclease, there was 15% spontaneous DNA fragmentation in mouse liver nuclei, 17% in P815 nuclei, and 14% in Yac-1 nuclei. The DNA fragmentation assays (described in Materials and Methods) were repeated at least twice and the variations were within ±5%.

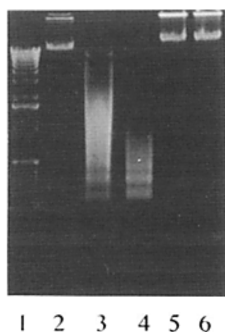

Figure 4. Electrophoresis of DNA isolated from mouse liver nuclei treated with $CaCl_2$, $MgCl_2$, and $ZnCl_2$. (Lane *1*) 1-kb DNA marker from GIBCO BRL; (lane *2*) untreated nuclei; (lane *3*) nuclei treated with 1.2 mM $CaCl_2$ and $MgCl_2$ for 1.5 h; (lane *4*) nuclei treated with the same concentration of Ca^{2+}/Mg^{2+} for 3 h; (lane *5*) nuclei pretreated with 100 µM $ZnCl_2$ for 1 h, followed by 1.2 mM $CaCl_2$ and $MgCl_2$ for 3 h; (lane *6*) 150 µM $ZnCl_2$ was used for pretreatment.

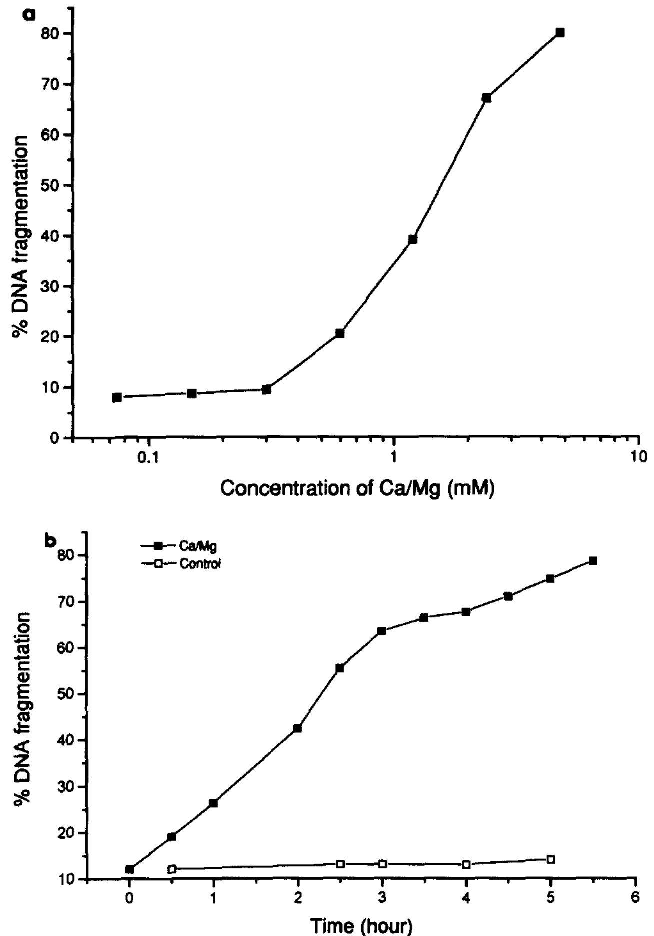

Figure 5. Dose and time dependence of DNA digestion induced by Ca^{2+}/Mg^{2+} in mouse liver nuclei, as measured by the DNA fragmentation assay. (*a*) Dose dependence of the effect of Ca^{2+}/Mg^{2+} on DNA fragmentation in mouse liver nuclei after a 3-h incubation. (*b*) DNA fragmentation induced by 1.2 mM $CaCl_2$ and $MgCl_2$ at different time points between 0 and 5.5 h (■). (□) DNA fragmentation in the absence of Ca^{2+}/Mg^{2+}. The DNA fragmentation assays (described in Materials and Methods) were repeated at least twice and the variations were within ±5%.

with 0.9 mM $CaCl_2$ and $MgCl_2$ for 3 h. The DNA fragmentation induced by $CaCl_2$ and $MgCl_2$ was completely inhibited by 100 µM $ZnCl_2$ (Fig. 4), but a typical pattern of chromatin condensation was still observed (Fig. 6 *d*). This suggests that these two processes could be triggered independently during apoptosis.

Valinomycin Induces Chromatin Condensation but not DNA Fragmentation in Intact Mouse Liver Cells. To further test the possibility that endonuclease activation may not suffice to induce chromatin condensation, we sought an example where chromatin condensation could be induced without DNA fragmentation in intact cells. In has previously been reported that valinomycin, a potassium ionophore, can induce traits characteristic of apoptosis in several cell lines (25, 40, 41). In agreement with these results, we observed that 100 µM valinomycin triggers DNA fragmentation in EL4, P815, and Yac-1 cells (data not shown). However, when mouse liver cells were ex-

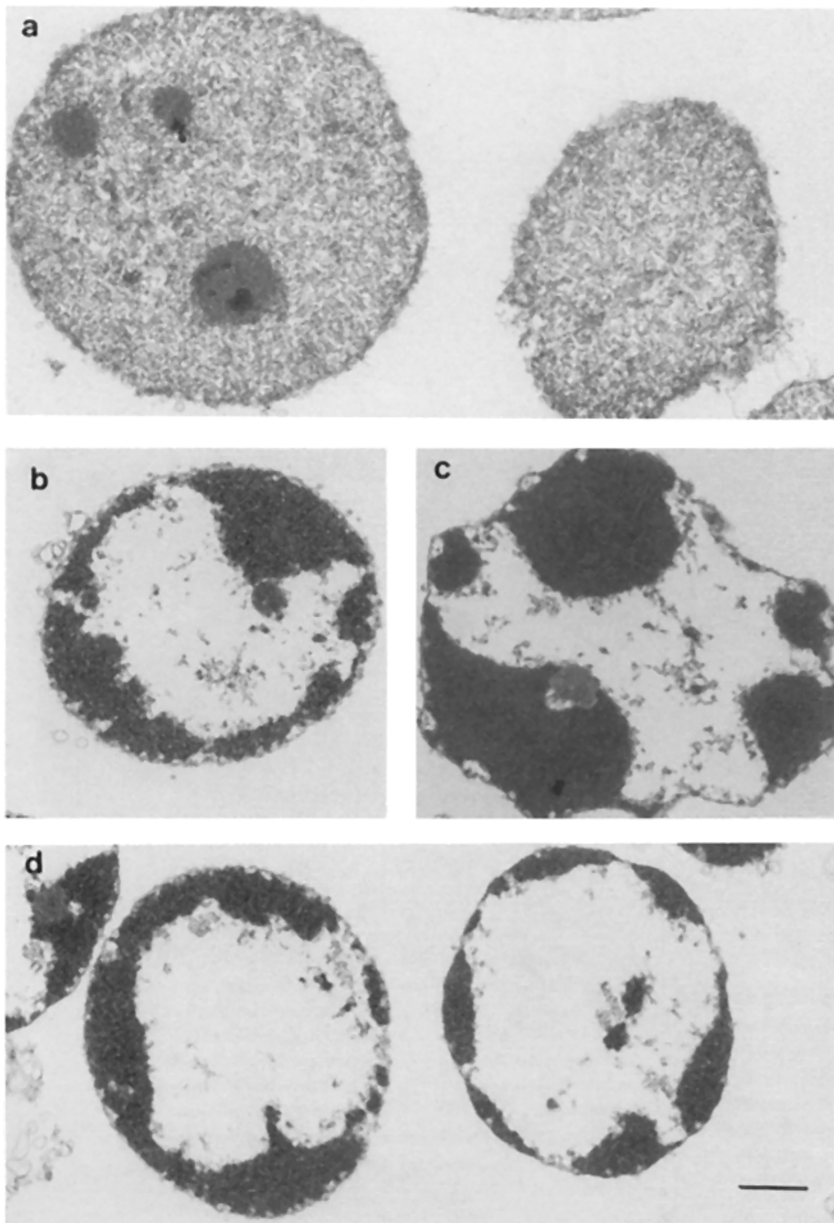

Figure 6. Nuclear changes in mouse liver nuclei resulting from treatment with Ca^{2+}/Mg^{2+} and $ZnCl_2$. Bar, 2 μm. (*a*) Untreated nuclei. (*b*) Chromatin condensation is observed in isolated mouse liver nuclei treated with 0.9 mM $CaCl_2$ and $MgCl_2$ for 3 h. (*c*) Stronger chromatin condensation appears after treatment with 1.2 mM $CaCl_2$ for 3 h. (*d*) Chromatin condensation is still present when nuclei are preincubated with 100 μM $ZnCl_2$ before incubation with 0.9 mM $CaCl_2$ and $MgCl_2$ for 3 h.

posed to the same concentration of valinomycin for periods between 4 and 24 h at 37°C, there was no DNA laddering observed at any time point (Fig. 7). Nonetheless, electron microscopy revealed typical chromatin condensation in the nuclei (Fig. 8, *b* and *c*) with cytoplasmic blebbing after a 4-h incubation with valinomycin. The cytoplasmic changes are likely due to secondary necrosis induced by valinomycin toxicity, which has been previously observed by our group (25). In contrast, liver cells incubated under the same conditions but in the absence of valinomycin demonstrated a normal nuclear morphology with intact cytosolic organelles (Fig. 8 *a*).

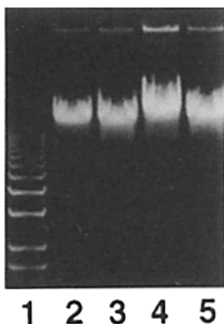

Figure 7. Absence of DNA fragmentation in mouse liver cells upon treatment with valinomycin. (Lane *1*) 1-kb DNA marker from GIBCO BRL; (lane *2*) untreated nuclei; (lanes *3–5*) nuclei treated with 100 μM valinomycin for 4, 12, and 24 h, respectively.

Discussion

Although the morphological characteristics of apoptosis have been described in detail, little is known about the cellular machinery underlying this process. In addition, although chromatin condensation and internucleosomal DNA laddering are two salient features of apoptotic cell death (21, 42), whether they are triggered by the same pathway or represent two independent events remains controversial (31, 35, 36).

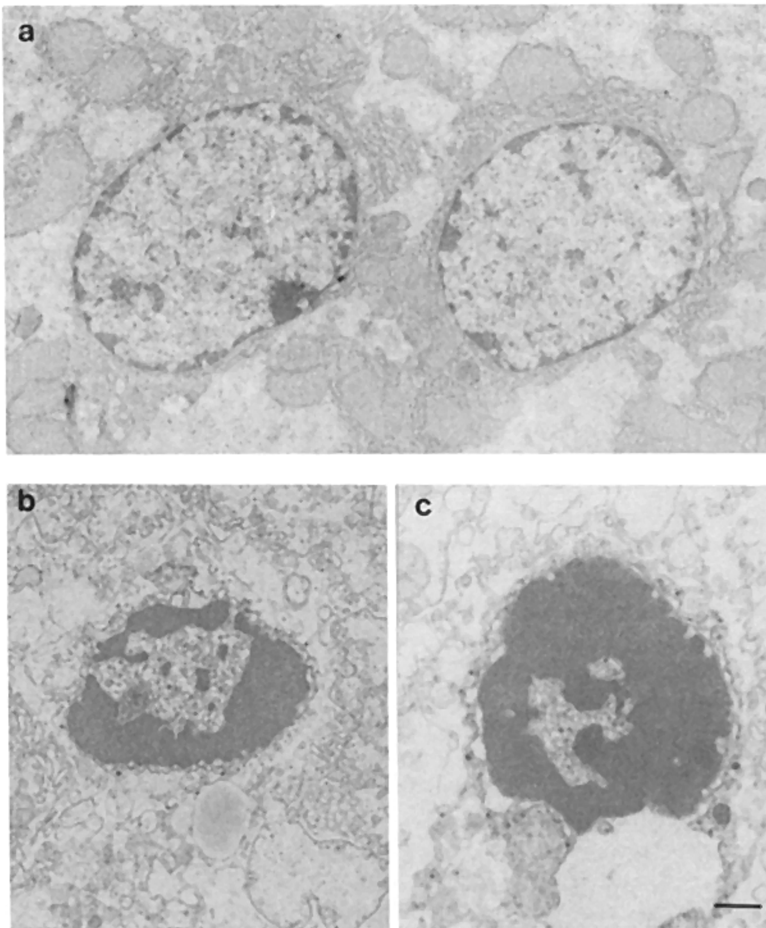

Figure 8. Apoptotic changes in mouse liver cells due to valinomycin treatment. (*a*) Normal morphology of cells after a 4-h incubation without valinomycin. (*b* and *c*) Chromatin condensation in mouse liver nuclei and bebbling in cytoplasm induced by 100 μM valinomycin after a 4-h incubation. Bar, 2 μm.

In 1980, Wyllie (21) observed that the morphological appearance of apoptosis in glucocorticoid-treated thymocytes was accompanied by DNA breakdown into integral multiples of about 180 bp. This led him to propose that steroids may induce an endonuclease with specificity much like that of micrococcal endonuclease, which cuts chromatin DNA in the linker regions between nucleosomes. More recently, Arends et al. (31) showed that micrococcal nuclease can induce both DNA fragmentation and chromatin condensation in isolated rat thymocyte nuclei. They concluded that selective activation of an endogenous endonuclease during apoptosis could be responsible not only for widespread chromatin cleavage but also for the major nuclear morphological changes.

To evaluate the nuclear changes that may be induced by micrococcal nuclease in a wider range of cell types, we used mouse liver nuclei and nuclei isolated from several tumor cell lines as model systems. This system is particularly useful as it bypasses the signal transduction pathways normally required to induce death in whole cells in response to extracellular signals. Furthermore, it has been proposed that protein synthesis may be required in some cases for apoptosis to occur (10, 43, 44). The use of isolated nuclei avoids these complications, as conditions in the nucleus can be directly manipulated and the effects upon DNA fragmentation and chromatin condensation ascertained.

In this study, no chromatin condensation was observed in the nuclei from any of the four cells tested after the nuclei were incubated with micrococcal nuclease at different concentrations or different time periods. These data show that digestion of genomic DNA into internucleosomal fragments alone does not cause concomitant collapse of chromatin structure, leading to chromatin condensation. For comparison, we attempted to test mouse thymocyte nuclei, but even in the normal thymocyte nucleus there were large amounts of condensed heterochromatin, making it difficult to identify specific chromatin condensation in treated nuclei. It is possible that previous observations of chromatin condensation in isolated thymocyte nuclei after treatment with micrococcal nuclease (31) may be due to erroneous identification of heterochromatin as chromatin condensation.

Several groups have shown that a Ca^{2+}/Mg^{2+}–dependent endonuclease exists in rat liver nuclei (39, 45) and mouse thymocyte nuclei (9). Recently, Ucker et al. (32) and Peitsch et al. (33) have independently reported that a DNase I–like endonuclease is involved in nuclear DNA fragmentation during apoptosis. We attempted to activate this endonuclease by altering the concentrations of Ca^{2+}/Mg^{2+} in the media. However, unlike micrococcal nuclease treatment, Ca^{2+}/Mg^{2+} triggered not only DNA fragmentation but also induced chromatin condensation (Fig. 6). Given our observa-

tion that micrococcal nuclease treatment alone does not cause chromatin condensation, it is possible that the effects of Ca^{2+}/Mg^{2+} may be due to the activation of other enzymes besides endonucleases, which may alter the structure of chromatin, resulting in its condensation.

In these experiments, millimolar concentrations of Ca^{2+}/Mg^{2+} have been used, whereas the cytosolic Ca^{2+} concentrations in thymocytes undergoing apoptosis have been found to be in the range of 400–700 nM (39). It would be useful to know which cellular factors contribute to the enhanced sensitivity of nuclei to submicromolar Ca^{2+} in intact cells. Alternatively, the calcium concentrations reported for apoptotic thymocytes may simply represent average cellular concentrations, which does not exclude the possibility that localized calcium gradients, with higher concentrations in the nucleus, may exist within the cell.

Cohen and Duke (9) have reported that glucocorticoid-mediated apoptosis in thymocytes may involve glucocorticoid activation of a calcium-dependent endonuclease, and they have shown that apoptosis could be prevented by the use of zinc, an endonuclease inhibitor. Likewise, zinc inhibited cell death in P815 tumor cells incubated with valinomycin (25). More recently, Cohen et al. (35) dissociated chromatin condensation from internucleosomal DNA fragmentation by treating intact thymocytes undergoing glucocorticoid-induced apoptosis with $ZnCl_2$. However as $ZnCl_2$ is known to be a nonspecific inhibitor of protein and RNA synthesis, and is a blocker of Ca^{2+} influx as well as an endonuclease inhibitor (9, 46, 47), it was not clear whether this was a direct effect upon endonucleases or involved nonspecific inhibition of cytosolic protein synthesis or Ca^{2+} influx. Our analysis of the effects of $ZnCl_2$ circumvents these problems through the use of isolated mouse liver nuclei, which lack the cytosolic targets for $ZnCl_2$ action, and suggests that $ZnCl_2$ does in fact directly inhibit endonucleases in isolated mouse liver nuclei (Fig. 4). Based on our observation that $ZnCl_2$ only inhibits DNA fragmentation without blocking the chromatin condensation induced by Ca^{2+}/Mg^{2+}, we conclude that endonuclease activation causes DNA fragmentation but is not necessarily responsible for chromatin condensation.

Valinomycin has been shown to induce apoptosis and increases in cytosolic Ca^{2+} levels in several tumor cell lines (25). However in mouse liver cells, treated with valinomycin, we observed an unusual form of cell death, morphologically similar to apoptosis but consisting of a mixture of nuclear apoptotic changes (chromatin condensation) and cytoplasmic necrotic changes. However, no DNA fragmentation was observed even up to 24 h. Other recent reports have also demonstrated the induction of apoptosis without DNA fragmentation in rat hepatocytes by okadaic acid (an inhibitor of phosphoprotein phosphatase 1 and 2A) and TGF-β1 (48, 49). This finding further supports the hypothesis that chromatin condensation may occur without activation of endonucleases during apoptosis.

In summary, we challenged isolated nuclei with micrococcal nuclease to mimic the genomic digestion due to endonuclease activation during apoptosis. We observed that micrococcal nuclease induced a DNA ladder pattern but no chromatin condensation. Using Ca^{2+}/Mg^{2+} to activate the Ca^{2+}/Mg^{2+}–dependent endonuclease in isolated liver nuclei, we were able to set off both DNA fragmentation and chromatin condensation. Nonetheless, the chromatin condensation was not due to endonuclease activity, since $ZnCl_2$ inhibited DNA fragmentation but failed to block the chromatin condensation induced by Ca^{2+}/Mg^{2+}. Similar results were obtained with whole liver cells, where we could trigger apoptotic-type morphological changes without DNA fragmentation after incubating the cells with valinomycin. Taken together, our results suggest that endonuclease activation is neither necessary nor sufficient to induce chromatin condensation in apoptosis, and they raise the possibility that DNA fragmentation and the nuclear structural changes may be caused by separate mediators in cells undergoing apoptosis.

We would like to thank Tellervo Huima-Byron and Howard Wynder at the New York Blood Center for their technical assistance in obtaining the electron micrographs; and Drs. Zanvil A. Cohn, John Gubbay, David Ucker, Svetlana Mojsov, Robert Darnell, and Leslie Leonard for critical reading of this manuscript.

This work was supported in part by grants from the National Institutes of Health (CA-47307 to J. D.-E Young), the American Cancer Society (CH425), and the American Heart Association, New York City affiliate. J. D.-E Young is a Scholar of the Leukemia Society of America.

Address correspondence to Dr. Dexter Y. Sun, Laboratory of Molecular Immunology and Cell Biology, The Rockefeller University, 1230 York Avenue, Box 280, New York, NY 10021-6399.

Received for publication 3 August 1993 and in revised form 5 October 1993.

References

1. Kerr, J.F.R., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* 26:239.
2. Shi, L., R.P. Kraut, R. Aebersold, and A.H. Greenberg. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med.* 175:553.
3. Duvall, E., and A.H. Wyllie. 1986. Death and the cell. *Immunol. Today.* 7:115.

4. Golstein, P., D.M. Ojcius, and J.D.-E Young. 1991. Cell death mechanisms and the immune system. *Immunol. Rev.* 121:29.
5. Sen, S., and M. D'Incalci. 1992. Apoptosis: biochemical events and relevance to cancer chemotherapy. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 307:122.
6. Arends, M.J., and A.H. Wyllie. 1991. Apoptosis: mechanisms and roles in pathology. *Int. Rev. Exp. Pathol.* 32:223.
7. Cohen, J.J. 1991. Programmed cell death in the immune system. *Adv. Immunol.* 50:55.
8. Zheng, L.M., A. Zychlinsky, C.-C. Liu, D.M. Ojcius, and J.D.-E Young. 1991. Extracellular ATP as a trigger for apoptosis or programmed cell death. *J. Cell Biol.* 112:279.
9. Cohen, J.J., and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132:38.
10. Wyllie, A.H., R.G. Morris, A.L. Smith, and D. Dunlop. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* 142:67.
11. Raff, M.C. 1992. Social controls on cell survival and cell death. *Nature (Lond.).* 356:397.
12. Vaux, D. 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc. Natl. Acad. Sci. USA.* 90:786.
13. Williams, L., and L. Bell. 1991. Asynchronous development of the rat colon. *Anat. Embryol.* 183:573.
14. Lewinson, D., and M. Silbermann. 1992. Chondroclasts and endothelial cells collaborate in the process of cartilage resorption. *Anat. Rec.* 233:504.
15. Furtwangler, J.A., S.J. Hall, and M.L. Koskinen. 1985. Sutural morphologenesis in the mouse calvaria: the role of apoptosis. *Acta Anat.* 124:74.
16. Colombel, M., C.A. Olsson, P.Y. Ng, and R. Buttyan. 1992. Hormone-regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle. *Cancer Res.* 52:4313.
17. Ferguson, D.J., and T.J. Anderson. 1981. Morphological evaluation on cell turnover in relation to the menstrual cycle in the "resting" human breast. *Br. J. Cancer.* 44:177.
18. Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkinson, and J.T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature (Lond.).* 337:181.
19. Cohen, J.J., and R.C. Duke. 1992. Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* 10:267.
20. Monti, D., L. Troinano, F. Tropea, E. Grassilli, A. Cossarizza, D. Barozzi, M.C. Pelloni, M.G. Tamassia, G. Bellomo, and C. Franceschi. 1992. Apoptosis-programmed cell death: a role in the aging process? *Am. J. Clin. Nutr.* 55:1208S.
21. Wyllie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (Lond.).* 284:555.
22. Sellins, K.S., and J.J. Cohen. 1987. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* 139:3199.
23. Schmid, D.S., J.P. Tite, and N.H. Ruddle. 1986. DNA fragmentation: manifestation of target cell destruction mediated by cytotoxic T-cell lines, lymphotoxin-secreting helper T-cell clones, and cell-free lymphotoxin-containing supernatant. *Proc. Natl. Acad. Sci. USA.* 83:1881.
24. Sellins, K.S., and J.J. Cohen. 1991. Cytotoxic T lymphocytes induce different types of DNA damage in target cells of different origins. *J. Immunol.* 147:795.
25. Ojcius, D.M., A. Zychlinsky, L.M. Zheng, and J.D.-E. Young. 1991. Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. *Exp. Cell. Res.* 197:43.
26. Kizaki, H., T. Tadakuma, C. Odaka, J. Muramatsu, and U. Ishimura. 1989. Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. *J. Immunol.* 143:1790.
27. Duke, R.C., and J.J. Cohen. 1986. IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. *Lymphokine Res.* 5:289.
28. Batistatou, A., and L.A. Greene. 1991. Aurintricarboxylic acid rescues PC12 and sympathetic neurons form cell death caused by nerve growth factor deprivation: correlation with suppression of endonuclease activity. *J. Cell Biol.* 115:461.
29. Trauth, B.C., C. Klas, A.M.J. Peters, S. Katzku, P. Moller, W. Falk, K.-M. Debatin, and P.H. Krammer. 1989. Monoclonal antibody–mediated tumor regression by induction of apoptosis. *Science (Wash. DC).* 245:301.
30. Evan, G.I., A.H. Wyllie, C.S. Gilbert, T.D. Littlewood, H. Land, M. Brooks, C.M. Waters, L.Z. Penn, and D.C. Hancock. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell.* 69:119.
31. Arends, M.J., R.G. Morris, and A.H. Wyllie. 1990. Apoptosis: the role of the endonuclease. *Am. J. Pathol.* 136:593.
32. Ucker, D.S., P.S. Obermiller, W. Eckhart, J.R. Apgar, N.A. Berger, and J. Meyers. 1992. Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes. *Mol. Cell. Biol.* 12:3060.
33. Peitsch, M.D., B. Polazar, H. Stephan, T. Crompton, H.R. MacDonald, H.G. Mannherz, and J. Tschopp. 1993. Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *EMBO (Eur. Mol. Biol. Organ.) J.* 12:371.
34. Duke, R.C., R. Chervanak, and J.J. Cohen. 1983. Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc. Natl. Acad. Sci. USA.* 80:6361.
35. Cohen, G.M., X.-M. Sun, R.T. Snowden, D. Dinsdale, and D.N. Skilleter. 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.* 286:331.
36. Tomei, L.D., J.P. Shapiro. and F.O. Cope. 1993. Apoptosis in C3H/10T1/2 mouse embryonic cells: evidence for internucleosomal DNA modification in the absence of double-strand cleavage. *Proc. Natl. Acad. Sci. USA.* 90:853.
37. McConkey, D.J., S. Orrenius, and M. Jondal. 1990. Cellular signalling in programmed cell death (apoptosis). *Immunol. Today.* 11:120.
38. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemistry.* 62:315.
39. Jones, D.P., D.J. McConkey, P. Nicotera, and S. Orrenius. 1989. Calcium-activated DNA fragmentation in rat liver nuclei. *J. Biol. Chem.* 264:6398.
40. Allbritton, N.L., C.R. Verret, R.C. Wolley, and H.N. Eisen. 1988. Calcium ion concentrations and DNA fragmentation in target cell destruction by murine cloned cytotoxic T lymphocytes. *J. Exp. Med.* 167:514.
41. Duke, R.C., P.M. Persechini, S. Chang, C.-C. Liu, J.J. Cohen, and J.D.-E Young. 1989. Purified perforin induces target cell lysis but not DNA fragmentation. *J. Exp. Med.* 170:1451.
42. Wyllie, A.H., J.F.R. Kerr, and A.R. Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251.
43. Lieberman, M.W., R.S. Verbin, M. Landay, H. Liang, E. Farber,

T.-N. Lee, and R. Starr. 1970. A probable role for protein synthesis in intestinal epithelial cell damage induced in vivo by cytosine arabinoside, nitrogen mustard, or X-irradiation. *Cancer Res.* 30:942.
44. Yamada, T., and H. Ohyama. 1988. Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis). *Int. J. Radiat. Biol.* 53:65.
45. Vanderbilt, J.N., K.S. Boom, and J.N. Anderson. 1982. Endogenous nuclease: properties and effects on transcribed genes in chromatin. *J. Biol. Chem.* 257:13009.
46. Waring, P., F.J. Kos, and A. Mullbacher. 1991. Apoptosis or programmed cell death. *Med. Res. Rev.* 11:219.
47. Lennon, S.V., and T.G. Cotter. 1991. The regulatory role of zinc in apoptosis in human tumor cell lines. *Biochem. Soc. Trans.* 20:78S.
48. Be, R., B.T. Ghertsen, O.K. Vintermyr, G. Houge, M. Lanotte, and S.O. Dskeland. 1991. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cell. *Exp. Cell Res.* 195:237.
49. Oberhammer, F.A., M. Pavelka, S. Sharma, R. Tiefenbacher, A.F. Purchio, W. Bursh, and R. Schulte-Hermann. 1992. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc. Natl. Acad. Sci. USA.* 89:5408.