Abstract. Apoptosis is the predominant form of cell death and occurs under a variety of physiological and pathological conditions. Cells undergoing apoptotic cell death reveal a characteristic sequence of cytological alterations including membrane blebbing and nuclear and cytoplasmic condensation. Activation of an endonuclease which cleaves genomic DNA into internucleosomal DNA fragments is considered to be the hallmark of apoptosis. However, no clear evidence exists that DNA degradation plays a primary and causative role in apoptotic cell death. Here we show that cells enucleated with cytochalasin B still undergo apoptosis induced either by treatment with menadione, an oxidant quinone compound, or by triggering APO-1/Fas, a cell surface molecule involved in physiological cell death. Incubation of enucleated cells with the agonistic monoclonal anti-APO-1 antibody revealed the key morphological features of apoptosis. Moreover, in non-enucleated cells inhibitors of endonuclease blocked DNA fragmentation, but not cell death induced by anti-APO-1. These data suggest that DNA degradation and nuclear signaling are not required for induction of apoptotic cell death.
that a cell nucleus and DNA fragmentation are not required for the initiation of apoptotic events.

**Materials and Methods**

**Cells and Reagents**

L929-APO-1 cells were derived from the murine fibrosarcoma cell line L929 after stable transfection with pEX-APO-1, an expression plasmid coding for human APO-1/Fas (22, 29). Cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 100 units of penicillin/ml and 0.1 mg streptomycin/ml. Expression of APO-1/Fas was verified by fluorescence immunochemistry and sensitivity towards the cytotoxic effects of anti–APO-1 (29). Anti-APO-1 monoclonal antibody (mouse IgG3) has been described in (34) and was used after purification by protein A affinity chromatography.

**Enucleation of Cells**

L929-APO-1 cells were enucleated essentially as described (19, 25). Briefly, 2 × 10⁶ cells were seeded in 25 cm² tissue flasks. 12 h later, the medium was exchanged against serum-free medium containing 10 µg/ml cytochalasin B (Sigma Chem. Co., Deisenhofen, FRG). For enucleation, cells were incubated for 90 min in the presence of cytochalasin B and then centrifuged inside the flasks in a Sorvall GSA rotor at 37°C and 25,000 g for 50 min. After centrifugation, cells were allowed to recover for 2 h in serum-containing medium without cytochalasin B and then treated with anti–APO-1 or menadione (Sigma Chem. Co.). Non-enucleated controls were treated with cytochalasin B without subsequent centrifugation.

**Light Microscopy**

Cells were washed twice with PBS and fixed in ice-cold methanol/ethanol (1:3) for 1 h. Fixed cells were stained for 30 min with propidium iodide (1 µg/ml) in PBS containing 0.1 mg/ml RNase. The specimens were analyzed under phase contrast and fluorescent light using a Zeiss Axiovert 35 microscope.

**Electron Microscopy**

L929-APO-1 cells were grown on THERMANOX coverslips (Nunc, Wisbaden, FRG) and enucleated in Sorvall tubes similarly as described above. Cells were fixed with 2.5% glutaraldehyde for 15 min in 50 mM cacodylate buffer, pH 7.4, containing 50 mM KCl and 2.5 mM MgCl₂. After three washing steps in buffer, cells were postfixed for 2 h with 2% osmium tetroxide in 25 mM plain cacodylate buffer and block-stained overnight with 0.5% uranyl acetate in water. Embedding and all other procedures were performed essentially as described (7).

**Cytotoxicity Assays**

Following enucleation, cells were treated with the indicated concentrations of anti–APO-1 or menadione in the presence or absence of actinomycin D (1 µg/ml). After 12 h, cells were stained with crystal violet or MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (18). The resulting formazan product was solubilized in 0.1% sodium dodecylsulphate, 10 mM HCl and measured spectrophotometrically at 540 nm.

**Gel Electrophoresis of Fragmented DNA**

Cells were seeded in 6-well plates at 1.5 × 10⁶ cells/well. 3 h later, the

![Figure 1](https://example.com/figure1.png)
cells were either left untreated or pretreated for 1 h with 300 \( \mu \)M aurintricarboxylic acid (ATA) (Sigma Chem. Co.) and then further incubated with 1.5 \( \mu \)g/ml anti-APO-1. For analysis of genomic DNA, cells were gently scraped off and collected together with the nonattached cells in the supernatant. Cells were lysed in NTE buffer (100 mM NaCl, 40 mM Tris-HCl, 20 mM EDTA, pH 7.4) containing 0.5% SDS and 0.2 mg/ml proteinase K. After overnight incubation at 37°C, DNA was extracted twice with phenol-chloroform and precipitated by ethanol. Samples were dissolved in TE buffer and digested for 2 h with 0.1 mg/ml RNAse A. DNA fragmentation was analyzed on a 1.8% agarose gel in the presence of 0.5 \( \mu \)g/ml ethidium bromide.

**Results and Discussion**

The requirement of DNA degradation for apoptosis was investigated in murine L929 fibrosarcoma cells. L929 cells represent a suitable model for analyzing the functional role of DNA digestion because they can easily be enucleated upon incubation with cytochalasin B (19, 25, 38). When L929-APO-1 cells, stably expressing human APO-1/Fas, were treated with anti-APO-1 antibodies, they displayed characteristic features of apoptosis within 30 min. First morphological alterations included the appearance of membrane blebbing and cytoplasmic condensation (Fig. 1 C). This was followed by the condensation of nuclear chromatin, which was most pronounced within 2–6 h after cell treatment (Fig. 1 D). Agarose gel electrophoresis further revealed extensive DNA digestion induced by anti-APO-1 (see Fig. 4 A). These events closely resembled apoptotic alterations induced by anti-APO-1 in other cell types (13, 34). To analyze whether typical apoptotic manifestations such as membrane blebbing and cytoplasmic condensation can also be induced in the absence of a cell nucleus, L929-APO-1 cells were enucleated. Cells were seeded in tissue flasks and treated with cytochalasin B. 90 min later, nuclei were removed by high speed centrifugation of the tissue flasks. As assessed by DNA staining with the karyophilic dye propidium iodide this procedure resulted in enucleation of about 80–90% of the cells (Fig. 2, A and B). Measurement of succinate dehydrogenase activity further revealed that cell viability was not seriously affected within 30 h after enucleation (data not shown). When the cytoplasts were treated with anti-APO-1, 2 h after expulsion of the nuclei, apoptotic alterations identical to the ones in cells with nuclei were observed. Membrane blebbing was induced in enucleated cells within 30 min after anti-APO-1 treatment as indicated by phase-contrast microscopy (Fig. 2 C). In addition, the enucleated cells became condensed and phase-bright similarly as observed in non-enucleated parent cells. Parallel staining of nuclear chromatin with propidium iodide revealed that cells undergoing apoptosis were indeed devoid of a nucleus (Fig. 2 D).

Apoptotic alterations in enucleated cells were also seen by electron microscopical analysis (Fig. 3, A and B). Cytoplasts treated with anti-APO-1 displayed vacuolization and mem-

**Figure 2.** Morphological changes induced by anti-APO-1 in enucleated cells. L929-APO-1 cells were seeded in tissue flasks as described in Fig. 1. 12 h later the medium was exchanged against serum-free medium containing 10 \( \mu \)g/ml cytochalasin B. For enucleation cells were incubated for 90 min in the presence of cytochalasin B and then centrifuged at 37°C and 25,000 g for 30 min. (A and B) Untreated cells; (C and D) anti-APO-1-treated cells: (C) Enucleated cells revealing membrane blebbing and cytoplasmic condensation are marked by an arrow. Bars, 20 \( \mu \)m.
brane-bound protrusions of the cytoplasm. The membrane blebs contained cytoplasmic material, ribosomes, and mostly dilated and swollen endoplasmic reticulum (Fig. 3 B). Typically for early stages of apoptosis, the ultrastructure of mitochondria was not significantly altered. Later stages ended in the complete fragmentation of the cytoplasts (data not shown).

To analyze whether anti-APO-1 treatment induced cytoxic effects after enucleation, cells were stained for succinate dehydrogenase activity, a known marker of mitochondrial function and cell viability (18). Enucleated and control cells (cells incubated with cytochalasin B without subsequent centrifugation) were treated for 12 h with anti-APO-1. As shown in Table I A, cell viability was markedly reduced by anti-APO-1 in non-enucleated and enucleated cells. Interestingly, the transcriptional inhibitor actinomycin D potentiated cytotoxicity of anti-APO-1 in control cells but not in cells devoid of a nucleus.

Apoptosis can be induced by a variety of diverse agents. To investigate whether a cell nucleus was also not required for other forms of apoptosis, experiments with menadione were performed. Menadione is a quinone compound that undergoes redox cycling leading to continuous formation of superoxide anions (33). Treatment with menadione has been shown to result in oxidative cell death resembling apoptosis (11, 16). In L929-APO-1 cells, apoptosis was induced by 25–100 mM menadione within 12 h. As shown in Table I B, there was no significant difference in sensitivity towards menadione in enucleated and non-enucleated cells. Therefore, these observations clearly indicate that the presence of a nucleus and consequently endonucleolytic DNA degradation is not required to transmit apoptotic signals. Both, morphological features of apoptosis and cytotoxic effects mediated by APO-1/Fas or by the oxidant menadione were observed in enucleated cells.

These conclusions were further supported by experiments with an endonuclease inhibitor. A nonlysosomal Ca²⁺/Mg²⁺-dependent endonuclease assumed to be involved in apoptotic DNA cleavage is inhibited by zinc ions (6, 8) or ATA (17). Although the action of ATA is not specific, treatment with ATA may provide evidence for a dependency of cell killing on DNA fragmentation. When cells were incubated with anti-APO-1, agarose gel electrophoresis of genomic DNA revealed the typical DNA ladder composed of 180-bp integers (Fig. 4 A). Preincubation with 300 μM ATA followed by anti-APO-1 treatment inhibited the formation of a DNA ladder and fragmentation of DNA into 180-bp multimers (Fig. 4 A), although it cannot be excluded that fragmentation of DNA into high molecular weight fragments (e.g., of 50 kb) might still have occurred (21). However, under these conditions APO-1-mediated cytotoxicity as assessed by cell morphology and viability remained virtually unaffected (Fig. 4 B). Thus, inhibition of anti-APO-1-induced endo-

Table I. Cytotoxic Effects of Anti-APO-1 (A) and Menadione (B) on Enucleated and Non-enucleated Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Viability (OD₅₇₀,₅₅₀)</th>
<th>Medium</th>
<th>Anti-APO-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control cells</td>
<td>1.18 ± 0.11</td>
<td>0.52 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Control cells + Act D</td>
<td>0.97 ± 0.10</td>
<td>0.25 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Enucleated cells</td>
<td>0.88 ± 0.15</td>
<td>0.34 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Enucleated cells + Act D</td>
<td>0.80 ± 0.13</td>
<td>0.31 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Medium</td>
<td>Menadione</td>
<td>Menadione</td>
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<tr>
<td>----------------------</td>
<td>--------</td>
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</tr>
<tr>
<td>25 μM</td>
<td>0.92 ± 0.06</td>
<td>0.68 ± 0.09</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.78 ± 0.15</td>
<td>0.42 ± 0.03</td>
<td>0.16 ± 0.03</td>
</tr>
</tbody>
</table>

L929–APO-1 cells were treated and enucleated as described in Fig. 2. Controls were incubated with cytochalasin B but not enucleated by centrifugation. Cells were treated for 12 h with 1.5 μg/ml anti-APO-1 (A) in the presence or absence of 1 μg/ml actinomycin D (Act D) or with the indicated concentrations of menadione (B). Cell viability was determined by staining with MTT and recorded spectrophotometrically at 540 nM. OD values are given as mean ± SEM of triplicates from a representative experiment.
nuclease activation efficiently prevented DNA degradation, but had no protective effect on cell viability.

In conclusion, these experiments demonstrate that DNA fragmentation is not the critical event in apoptosis induced by the physiological inducer APO-1/Fas or the nonphysiological oxidant menadione. Whether this observation also holds true for apoptotic events induced by other initiators of apoptosis remains to be established. At least TNF-mediated cytotoxic effects cannot be prevented by inhibitors of endonuclease activity, but did not rescue these cells from corticoid-induced cell death (3). Consistent with this, it has been described that cytotoxic T cell-mediated apoptosis was also induced in fibroblast clones lacking detectable endonuclease activity (35). Thus, the present study reveals that DNA degradation may be characteristic but not necessary for the sequence of events leading to apoptosis. Nonetheless, DNA degradation may facilitate disintegration of the cell and thereby support phagocytic removal of dead cells. Our observations, therefore, are not compatible with the widely accepted dogma that internucleosomal DNA fragmentation is a mandatory event for apoptotic cell death. The experiments with enucleated cells show that induction of apoptosis must involve cytoplasmic steps rather than endonuclease activation in the nucleus.

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Note Added in Proof. During typesetting of this manuscript a study appeared showing that staurosporine-induced apoptosis can also be seen in cytoplasts derived from a fibroblast and oligodendrocyte cell line. (Jacobson et al. 1994. EMBO Eur. Mol. Biol. Organ. J. 13:1899-1910.)

Figure 4. The effect of the endonuclease inhibitor aurintricarboxylic acid (ATA) on anti-APO-1-induced DNA fragmentation (A) and cell survival (B). (A) L929-APO-1 cells were seeded in 6-well plates at 1.5 × 10⁶/well. 3 h later cells were either left untreated (lanes 1 and 2) or pretreated for 1 h with 300 μM ATA (lanes 3 and 4) and then incubated for further 12 h with 1.5 μg/ml anti-APO-1 (lanes 2 and 4). After lysis of the cells, DNA was extracted with phenol-chloroform-isoamylalcohol, precipitated by ethanol, and dissolved in TE buffer with 0.1 mg/ml RNase A. DNA fragmen-
tation was analyzed on a 1.8% agarose gel in the presence of ethidium bromide. (B) Cytotoxicity assay: cells were seeded at 3 x 10⁴/well. 3 h later cells were either left untreated or pretreated for 1 h with 300 μM ATA or medium were added in a 50 μl vol. Following 1 h of further incubation, cells were treated with 1.5 μg/ml anti-APO-1 in the presence of 1 μg/ml actinomycin D. Cell survival was determined after 12 h by staining with crystal violet. Values represent the mean of triplicates + SEM out of three experiments.

References


