Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy

Wilfried Bursch1,2, Adolf Ellinger1, Harald Kienzl, Ladislaus Türök, Siyaram Pandey2, Marianna Sikorska2, Roy Walker2 and Rolf Schulte Hermann

Institut für Tumobiologie-Krebsforschung der Universität Wien, Borschkegasse 8a, A-1090 Wien, 1Histologisch-Embryologisches Institut der Universität Wien, Ordinariat II, Schwarzenbergstrasse 17, A-1090 Wien, Austria and 2Institute for Biological Sciences, National Research Council, Bldg M54, Montreal Road, Ottawa, Canada K1A 0R6

1To whom correspondence should be addressed

Active cell death in hormone-dependent cells was studied using cultured human mammary carcinoma cells (MCF-7) treated with the anti-estrogens (AEs) tamoxifen (TAM), 4-hydroxy-tamoxifen (OH-TAM) or ICI 164 384 (10^-9-10^-6 M) as a model. The following results were obtained. (i) In untreated MCF-7 cells a wave of replication occurred in the first 5 days of culture. All three AEs caused a dose-dependent inhibition of cell replication. (ii) TAM and OH-TAM at 10^-9 M, but not ICI 164 384, caused lytic cell death (necrosis) within 24 h, which was not inhibited by estradiol (10^-9-10^-4 M). (iii) Lower concentrations of TAM or OH-TAM (up to 10^-6 M) or ICI 164 384 induced a more gradual appearance of cell death beginning at day 3. This type of cell death was inhibited by estradiol (10^-6 M), indicating its active nature. (iv) Nuclei showed two distinct patterns of alteration: (a) apoptosis-like condensation and fragmentation of chromatin to crescent masses abutting the nuclear envelope; (b) condensation of the chromatin to a single, pyknotic mass in the center of the nucleus, detached from the nuclear envelope. Quantitative histological evaluation revealed the predominance of pyknosis. (v) Biochemical DNA analysis revealed that only a relatively small amount of the total DNA was finally degraded into low molecular weight fragments (20 kb and less). (vi) Active cell death, with both apoptotic and pyknotic nuclear morphology, was associated with extensive formation of autophagic vacuoles (AV). 3-Methyladenine, a known inhibitor of AV formation, partially prevented cell death as detected by nuclear changes. (vii) ICI 164 384 was about 10 times more effective than TAM or OH-TAM at inhibiting DNA synthesis, but had equal potency in inducing active cell death. It is concluded that AEs have anti-proliferative and anti-survival effects on MCF-7 human mammary cancer cells in culture. These two effects are under separate control because they differ by kinetics, dose dependence and sensitivity to the various AEs. Active cell death in MCF-7 cells seems to be initiated by autophagy, in contrast to concepts of apoptosis, and thus corresponds to autophagic/lysosomal or type II death as previously defined. This may be important because of biochemical and molecular differences between these various subtypes of active cell death.

Introduction

Active cell death (ACD*), i.e. apoptosis and other subtypes of ACD (see below), are under the control of growth regulating hormonal factors (1-6). Estrogens are important growth stimulators for mammary gland and other target tissues, including estrogen-dependent tumors (6-12). Estrogen withdrawal by ovariectomy causes regression and ACD in these tissues (4,7,13). Trophic effects of estrogens can also be reversed by estrogen antagonists. Among these, tamoxifen (TAM) is one of the most successful drugs in the treatment of human cancer, although most tumors eventually develop resistance to anti-estrogen (AE) treatment (14-16). In recent years several other AEs have been found and tested for anti-tumoral activity (14,16-18). These AEs consistently exhibit an anti-proliferative potency, however, relatively little is known about their capacity to induce death of tumor cells (6,19-22). To understand the mechanism of therapeutic effects of AEs it would be important to know whether and to what extent they induce ACD in cancers and, if so, which type of active death is involved.

ACD is now widely equated with apoptosis, a type of cell death characterized by specific morphological features: shrinkage of cytoplasm, condensation and fragmentation of chromatin to pieces abutting the nuclear membrane, phagocytosis of cell residues by neighboring cells (heterophagy) (1,23). However, some studies suggest the occurrence of additional types of ACD, with different morphology and biochemistry (4,24-27). These include type II or autophagic/lysosomal and type III cell death, the latter associated with disintegration of cells without involvement of the lysosomal system (24,25). These types of cell death were described as a form of programmed cell death during embryonic development and in several insect tissues during metamorphosis (26-29). Discrimination between morphologically different types of cell death should be important because of underlying differences in biochemical and molecular events leading to cell death; their elucidation may offer new targets for anti-tumor drugs.

In the present study we have used the human mammary cancer cell line MCF-7, which is known to grow estrogen-dependently and is widely used to study AE effects (for reviews see 9,14). Two reports have described the induction of apoptosis by AEs in this cell line (19,20). During detailed studies on AE effects in MCF-7 cells we confirmed the occurrence of cell death. However, only a minority of dying cells showed the typical morphological picture of apoptosis. Rather, as an early and predominant characteristic of cell death we noted the appearance of autophagic vacuoles (AVs) in the cytoplasm. The relevance of autophagy was supported by

*Abbreviations: ACD, active cell death; TAM, tamoxifen; AE, anti-estrogen; AVs, autophagic vacuoles; DMEM, Dulbecco's modified Eagle's medium; E2, estradiol; DCC, dextran-coated charcoal; DCC-FCS, DCC-stripped fetal calf serum; FCS, fetal calf serum; OH-TAM, 4-hydroxy-tamoxifen; PFOE, pulsed field gel electrophoresis; CAGE, conventional gel electrophoresis; LMP, low melting point; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.

†Dedicated to Emeritus Professor Dr. W. Koransky, Institut für Toxikologie und Pharmakologie, Marburg, Germany, on his 75th birthday.
Fig. 1. DNA synthesis and DNA content of MCF-7 cultures treated with TAM, 4-OH-TAM and ICI 164 384 and with E<sub>2</sub>. (A) [<sup>3</sup>H]Thymidine incorporation into DNA. (B and C) DNA content per culture dish. In (A) and (B) AE was administered alone on day 1 (arrow). In (C) 10<sup>-9</sup> M E<sub>2</sub> was administered along with AE on day 1 (arrow). O, Control; AE concentration: ▲, 10<sup>-8</sup> M; *, 10<sup>-7</sup> M; ●, 10<sup>-6</sup> M; ■, 10<sup>-5</sup> M. Note that [<sup>3</sup>H]thymidine incorporation into DNA was not determined in cultures treated with 10<sup>-8</sup> M AE. Dotted lines in (d)-(i), controls as given in (a)-(c). Means of three experiments are given. Vertical bars indicate SD, smaller than symbols where not shown. Symbols for statistical significance are not given for the sake of clarity of the figure.

Table I. Effect of E<sub>2</sub> (10<sup>-9</sup> M) on mitosis in OH-TAM- and ICI 164384 (10<sup>-6</sup>M)-treated cell cultures

<table>
<thead>
<tr>
<th>Day of investigation</th>
<th>Day of AE addition</th>
<th>Day of E&lt;sub&gt;2&lt;/sub&gt; addition</th>
<th>Treatment</th>
<th>Control</th>
<th>OH-TAM</th>
<th>OH-TAM + E&lt;sub&gt;2&lt;/sub&gt;</th>
<th>ICI 164384</th>
<th>ICI 164384 + E&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Control</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.2*</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>OH-TAM</td>
<td>1.1 ± 0.4</td>
<td>0.6 ± 0.3*</td>
<td>0.2 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3</td>
<td>OH-TAM + E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.9 ± 0.5</td>
<td>0.7 ± 0.4*</td>
<td>2.9 ± 0.9*</td>
<td>0.5 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>5</td>
<td>ICI 164384</td>
<td>2.7 ± 0.3</td>
<td>1.2 ± 0.1*</td>
<td>1.6 ± 0.3*</td>
<td>0.1 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5</td>
<td>ICI 164384 + E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.3 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>1.0 ± 0.5*</td>
<td>0.3 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

AE, anti-estrogen, administered on day 1 of the experiment; E<sub>2</sub>, estradiol, administered either along with AE (day 1 of the experiment), 2 or 4 days after AE treatment (day 3 or 5 of the experiment respectively). On the 'day of investigation' the cell cultures were processed for morphological analysis as described in Materials and methods. *P < 0.05, AE versus control; †P < 0.05, AE versus AE + E<sub>2</sub>. Numbers, mitoses as percentage (± SD) of non-dividing, intact cells.

the observation that 3-methyladenine, a known inhibitor of autophagy (30), largely prevented active death of MCF-7 cells. Thus, AEs seem to induce type II cell death in MCF-7 cells. Furthermore, we compared the effects of different AEs on cell death in relation to their anti-proliferative action. The results suggest that the induction of cell death can be separated from inhibition of cell proliferation.

Materials and methods

Materials

MCF-7 human breast cancer cells were kindly provided by Dr Otto (University of Regensburg, Germany). Dulbecco's modified Eagle's medium (DMEM) without phenol red was from Gibco (Meckenheim, Germany), plastic culture dishes from Falcon (Becton Dickinson Labware). The following reagents were purchased from Serva (Heidelberg, Germany): Trition-X-100, NADH (disodium...
Twenty four hours later the cells were treated with TAM ([a-(4-fi-N-3 3H]thymidine (20 Ci/mmol) was from New England Nuclear (Vienna, Austria), high strength analytical grade agarose was obtained from Fluka (Switzerland). The AEs were a gift from Zeneca Pharmaceuticals (Macclesfield, UK).

Preparation of dextran-coated charcoal (DCC) and DCC-striped fetal calf serum (DCC-FCS)

Charcoal was activated at 120°C overnight, followed by agitating in solution 1 (1 mM Tris, 1 mM EDTA, pH 7.6, 1:4 w/v) at room temperature for 10 min. After allowing the charcoal particles to sediment for 15 min, the supernatant was removed and the charcoal particles were treated in the same way five times with solution 2 (solution 1 plus 1% dextran). Heat-inactivated fetal calf serum (FCS) was agitated in DCC (mol. wt ~500 000) for 5 min, centrifuged at 8000 g, and sterilized by filtration through a cellulose membrane (pore size 20 nm). The cell pellet was resuspended in 0.25 ml of the above buffer and DCC-FCS was stored at ~20°C until use.

Cell culture

MCF-7 cells were cultured in DMEM without phenol red supplemented with 10% FCS, L-glutamine (300 mg/l), bovine insulin (100 IU/ml) and HEPES (10 mM) (designated ‘DMEM-F10’). Cells growing as a monolayer were kept at 37°C in a humid atmosphere in the presence of 5% CO2. Cell harvesting was performed using trypsin/EDTA (0.05%/0.02%). To obtain a single cell suspension, cells were further separated by the application of a syringe with a 22 gauge needle.

Seven days before the beginning of an experiment cells were steroid-withdrawn according to Bardon et al. (19). Cells were plated on glass coverslips in plastic dishes (diameter 35 mm) at a density of 7.5 x 10^3/cm^2 and the culture medium was replaced by DMEM-D3 (DMEM without phenol red containing 3% DCC-FCS, 300 mg/l L-glutamine and 10 mM HEPES). Twenty four hours later the cells were treated with TAM ([a-(4-fi-N-dimethylaminoethoxy)phenyl-a'-ethyl-trans-stilbene], 4-hydroxytamoxifen (TAM) or ICI 164384 (N-(4-hydroxy-a'-ethyl-trans-stilbene), OH-TAM) or ICI 164384 (N-(4-hydroxy-a'-ethyl-trans-stilbene). OH-TAM) or ICI 164384 (N-(4-hydroxy-a'-ethyl-trans-stilbene). OH-TAM)) or ICI 164384 (N-(4-hydroxy-a'-ethyl-trans-stilbene). OH-TAM)) or ICI 164384 (N-(4-hydroxy-a'-ethyl-trans-stilbene). OH-TAM). Estradiol, dextran (mol. wt ~500 000) and Soluene 200 were purchased from BioRad (Richmond, CA) and cellulose filters were from Whatman (Maidstone, UK). Cell culture media were from Gibco (BRL, Burlington, MA), a 1 kb DNA ladder and a 123 bp DNA ladder (Gibco BRL, Burlington, MA) were used as size markers. The gels were stained with ethidium bromide, placed on a transilluminator and photographed on Polaroid MP4 film. For quantitative analysis of degraded DNA cells were lysed by adding 100 μl ice-cold SDS buffer and 10 min later 900 μl buffer (0.1% SDS, 1 mM EDTA, 0.1 mM Tris, pH 7.4) Samples were allowed to stand overnight before measurement Aliquots of 800 μl of each sample was used for quantitative DNA determination using the fluorescent dye Hoechst H-33258; calf thymus DNA (7 μg/ml) was taken as a reference standard. The remaining 200 μl were taken for measurement of incorporated [3H]thyminde as described elsewhere (32).

DNA sample processing for pulsed field agarose (PFGE) and conventional (CAGE) gel electrophoresis

In order to avoid DNA shearing during sample handling, cellular samples were immobilized in low melting point (LMP) agarose plugs before deproteinization. Approximately 2-3 x 10^6 cells were harvested, washed once with 15 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM EDTA, 0.5 mM spermine, 0.15 mM spermine, 60 mM KCl, 15 mM NaCl buffer and pelleted at 700 g for 5 min at 4°C. The cell pellet was resuspended in 0.25 ml of the above buffer and mixed with an 0.25 ml aliquot of molten 1.5% (w/v) LMP agarose (at 37°C) and 100 μg proteinase K. The mixture was transferred immediately to a 1 ml syringe to solidify. The agarose plug was removed from the syringe and incubated overnight at 37°C in 3 ml 10 mM Tris-HCl, pH 9.5, 25 mM EDTA, 1 mM EDTA, 10 mM NaCl buffer, 400 μg proteinase K and 1% (w/v) laurylsarcosine. The plug was subsequently washed in 10 mM Tris-HCl, pH 8.0, 10 mM EDTA buffer and stored at 4°C. Equal length slices (~4 mm, equivalent to 0.5-0.7 x 10^6 cells) of the agarose plugs were loaded on an 0.8% agarose gel in 0.89 M Tris, 0.089 M boric acid, 25 mM EDTA, pH 8-8.5, the wells were sealed with 1.5% (w/v) LMP agarose and PFGE was carried out using a Q-life Autobase Electrophoresis System (Kingston, Ontario, Canada) as described by Walker et al. (33).

Small DNA fragments which leaked from the plugs during the deproteinization step were precipitated with cold 80% ethanol. The pellet was resuspended in 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and treated with RNase A followed by proteinase K. DNA was precipitated again with ethanol and analyzed by 0.8% CAGE. A 1.5A DNA ladder (New England Biolabs, Beverly, MA), a 1 kb DNA ladder and a 123 bp DNA ladder (Gibco BRL, Burlington, MA) were used a size markers. The gels were stained with ethidium bromide, placed on a transilluminator and photographed on Polaroid MP4 film. For quantitative analysis of degraded DNA cells were lysed for 30 min in 10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0, and centrifuged at 27 000 g. The LDH activity in the medium found before Triton X-100 addition the medium was performed according to Bergmeyer (34).

Table II. Inhibition of growth and induction of active cell death in MCF-7 cells by different AEs

<table>
<thead>
<tr>
<th>Dose</th>
<th>DNA synthesis (% inhibition)</th>
<th>DNA content (% inhibition)</th>
<th>Active cell death (% increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAM</td>
<td>OH-TAM</td>
<td>ICI 164384</td>
<td>TAM</td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>16</td>
<td>22</td>
<td>49</td>
</tr>
<tr>
<td>10^{-7} M</td>
<td>46</td>
<td>55</td>
<td>57</td>
</tr>
<tr>
<td>10^{-6} M</td>
<td>78</td>
<td>82</td>
<td>91</td>
</tr>
</tbody>
</table>

Growth inhibition by AEs is expressed as reduction in increase of DNA synthesis (day 1 versus day 4, see Figure 1) and DNA content (average of plateau beyond day 6 versus day 1, see Figure 1) in uninhibited cells. The rate of active cell death on day 10 as exemplified by the predominant nuclear pyknosis is expressed as a percentage of that of controls on day 10 (see Figure 6).

**P < 0.05, **P < 0.01 days 5-10 versus day 4.

The means of three experiments are given.

Fig. 2. DNA content of MCF-7 cultures treated with OH-TAM on day 4. Dotted line, controls as given in Figure 1b. OH-TAM: △, 10^{-6} M; ★, 10^{-5} M; ●, 10^{-6} M, ■, 10^{-5} M. Means of three experiments are given. Vertical bars indicate SD, smaller than symbols where not shown. 10^{-3} M, **P < 0.05, **P < 0.01 days 5–10 versus day 4.
Fig. 3. Ultrastructural features of cell death in MCF-7 cultures after AE treatment. (a) Control, day 7. The plasma membrane exhibits extended areas with microvilli (→); the cytoplasm typically shows multiple polyribosomes (arrowheads) and prominent Golgi regions (G). (b) 10^{-5} M TAM, day 1. The cell surface is rounded without microvilli. The cytoplasm shows multiple empty vacuoles and some AVs (→). Golgi stacks are not found. The chromatin is irregularly condensed, the nucleolus fragmented (arrowhead). (c) 10^{-6} M TAM, day 7. The nucleus appears normal, in the cytoplasm several AVs are obvious (→). (d) TAM 10^{-6} M, day 7. Ribbons of condensed chromatin are detached from the nuclear envelope. Numerous AVs (→) and prominent Golgi regions (G) mark the cytoplasm.

Morphological procedures
For light microscopy and quantitative analysis whole mount cells attached to glass coverslips were rinsed twice with phosphate-buffered saline (PBS), fixed with ice-cold paraformaldehyde (3% in PBS) for 10 min and washed twice with distilled water. After drying the cells were stained for 10 min with a freshly prepared solution of H-33258 in PBS (3 mg/ml, pH 5) and incubated for 5 min in citrate buffer (0.1 M sodium citrate, pH 3.0) in the dark. Thereafter cells were washed once with distilled water, once with ethanol and the glass coverslips were mounted on slides using Moviol as a mounting medium. The number of dead cells (for morphological description see Results) and mitoses were determined by scoring 9000 cells per time point (1000 cells in each of triplicate cultures, three experiments). The numbers of mitoses and dead cells were expressed as a percentage of the total number of intact cells scored.

For electron microscopical investigations, cells were harvested by trypsinization, washed twice with PBS and fixed with ice-cold glutaraldehyde (3% in 0.1 M cacodylate buffer, pH 7.4) for 30 min. After washing in PBS the cells were post-fixed in OsO_{4} embedded in Epon and 0.1 μm thin sections were stained with uranylacetate/lead citrate (Fluka) and viewed in a Philips EM 400 electron microscope. For light microscopical investigation, 1 μm sections were stained with toluidine blue.

Cells detached from the substrate were collected from the medium by centrifugation at 250 g for 5 min. For electron microscopical investigation, cells were fixed with ice-cold glutaraldehyde and processed as described above.

For quantitative light microscopical analysis unfixed cells were resuspended in trypan blue solution (0.25% in PBS) and counted in a hemocytometer.

Statistics
Each experiment was performed at least three times. In figures means ± SD are shown, for statistical analysis Student’s t-test was used. A comprehensive statistical analysis of the results presented in this paper are given in Kienzl (35).

Results
Effect of TAM, 4-OH-TAM and ICI 164 384 on growth of MCF-7 cells
In control cultures, an increase in DNA synthesis as measured by [3H]thymidine incorporation occurred with a sharp peak on day 4 after seeding. Subsequently [3H]thymidine incorporation decreased and remained at a low level until termination of the
experiment on day 10 (Figure 1a). Correspondingly, the DNA content of the cultures increased ~4-fold within 5 days and remained constant thereafter (Figure 1b). Mitotic activity rose until day 4 and declined again subsequently (Table I).

The AEs TAM, OH-TAM and ICI164 384 were administered to the culture medium 24 h after seeding (day 1). TAM at 10^{-7} and 10^{-6} M caused a significant, dose-dependent inhibition of DNA synthesis (Figure 1a), reducing the increase in DNA content. The latter effect became manifest on day 5 after 10^{-8} M TAM, but on day 3 after 10^{-6} M TAM (Figure 1b). Increasing the TAM dose to 10^{-5} M caused a complete block of ^{3}H\text{thymidine incorporation, associated with a complete loss of DNA from the plates. Essentially the same findings were made with OH-TAM (Figure 1d and e). These results suggest that TAM or OH-TAM up to 10^{-6} M exert a cytostatic effect, whereas at 10^{-5} M they cause cytostasis and cytolysis. This conclusion was confirmed in a further experiment when 10^{-5} M OH-TAM was added on day 4. OH-TAM again induced a rapid loss of DNA, while 10^{-8}…10^{-6} M showed only a marginal effect on DNA content (Figure 2). This was anticipated, because the cytostatic effect should not become manifest when the rate of DNA synthesis was beyond its peak.

ICI 164384 was tested at the same concentrations as TAM and OH-TAM (Figure 1g and h). It also caused a dose-dependent inhibition of ^{3}H\text{thymidine incorporation and of DNA increase. In contrast to TAM or OH-TAM, at 10^{-5} M ICI 164384 did not result in a rapid loss of DNA (Figure 1h). Furthermore, ICI 164384 was ~10 times more effective than TAM in inhibiting DNA synthesis (Table II), similar to previous observations (36). The increase in mitotic activity was blocked by ICI 164 384 as well as by OH-TAM (Table I).

The addition of E\textsubscript{2} (10^{-9} M) along with up to 10^{-6} M TAM or OH-TAM completely antagonized their growth inhibition as detected by DNA content (Figure 1c and f) and mitotic
The nuclei in control cells are characterized by prominent nucleoli; in the cytoplasm, organelles remain inconspicuous. Mitosis (b) TAM 10⁻² M, day 1. The cells are generally rounded up, clusters of vacuoles are visible (>). (c) TAM 10⁻⁶ M, day 7. In the center of the picture different stages in the formation of pyknotic nuclei are to be seen (arrowheads). Most cells show numerous AVs (>). (d) TAM 10⁻⁶ M, day 7. Pieces of crescent chromatin attached to nuclear membrane (arrowheads). →, AVs. Bars 10 µm.

Activity assay (Table I). E₂ addition 2 or 4 days after AE treatment resulted in reversion of the OH-TAM effect on mitotic activity (Table I). E₂ did not prevent the rapid loss of DNA after 10⁻⁵ M TAM or OH-TAM (Figure 1c and f). Furthermore, E₂ did not reverse ICI 164 384 (10⁻⁶ and 10⁻⁵ M)-induced growth inhibition as determined from DNA content (Figure 1i) or mitotic activity (Table I).

At concentrations up to 10⁻⁶ M AEs induced cell death, the appearance of which was delayed and did not result in significant DNA loss until termination of the experiment. The characteristics of the rapid and delayed types of cell death are described below.

Morphology of cell death in AE-treated cells

Electron microscopical investigation of control cultures revealed cells characterized by numerous cytoplasmic polyribosomes and pronounced Golgi fields with secretory vesicles; signs of autophagy were seen only occasionally (Figure 3a). The plasma membrane showed numerous microvilli and the nuclei were lobed, with clearly visible nucleoli and heterochromatin attached to the nuclear envelope (Figure 3a). At the light microscopical level, in semithin sections stained with toluidine blue the cytoplasm appeared homogenous, the nuclei contained several nucleoli and some mitoses could be observed (Figure 4a).

After TAM numerous dead cells occurred, but their morphology was strikingly different at 10⁻⁵ M and at 10⁻⁶ M or lower. Within 1 day after 10⁻⁵ M TAM, electron microscopy revealed severe signs of cell degeneration, such as extensive vacuolization of cytoplasm, disappearance of polyribosomes and disintegration of organelles and Golgi fields, as well as partial disruption of cell membrane with a loss of microvilli. The nuclei exhibited chromatin condensation to structures of irregular shape and disintegration of nucleoli (Figure 3b). Light microscopy showed that almost all cells were affected at this time point and that many cells were completely lysed (Figure 4b). ICI 164 384 at 10⁻⁵ M did not produce morphological signs of lytic cell death (data not shown).

Electron microscopical analysis after 10⁻⁶ M TAM was performed at 2, 4, 7 and 9 days after treatment (representative examples of morphological changes as observed at 7 days are...
looking cytoplasm and organelles were found; usually, the AVs contained membrane structures and were distributed throughout the cytoplasm (Figure 3c, d, e and f). Semi-quantitative analysis by light microscopy revealed that the vast majority of cells contained AVs perceivable as intensely stained round structures in toluidene blue stained sections (Figure 4c and d). Furthermore, cells with amorphous cytoplasm containing no visible polyribosomes or Golgi fields, but clusters of AVs, usually in close proximity to the nuclear membrane and associated with morphologically intact mitochondria, were found (Figure 3e).

Many of the cells displaying pronounced autophagy exhibited a normal looking nucleus (Figure 3c). In numerous other cells, however, one of two types of nuclear change was found. The first type showed slightly condensed chromatin detached from the nuclear membrane (Figure 3d) or extensive chromatin condensation to a single pyknotic mass in the central part of the nucleus; the nuclear membrane was well preserved (Figure 3e). Nuclear pyknosis was frequently found to be associated with amorphous appearance of the cytoplasm, suggesting that it reflects a late stage of cell death. In the second type of nuclear change chromatin was condensed to crescent masses abutting the nuclear membrane (Figure 3f). Also, small membrane-bound bodies attached to the nuclear envelope with highly condensed chromatin pieces of different sizes were found (Figure 3g). Many of the cells with altered nuclei of both types exhibited no microvilli and appeared of a more regular and rounded shape than cells with normal nuclei (Figure 3e–g). Taken together, electron and light microscopical examination of MCF-7 cells after up to 10⁻⁶ M TAM revealed that cell death is characterized by progressive autophagic degradation of cytoplasm, associated with nuclear changes either occurring as pyknosis or as during typical apoptosis (crescent nuclei). The morphological changes are considered to reflect a sequence in an individual cell, however, after AE treatment the cells enter the death pathway asynchronously and therefore all stages can be observed on an individual culture plate.

Finally, some dead cells detached from the substrate and lysed (a representative example of cell debris as found in the culture medium is shown in Figure 3h). For quantitative light microscopical analysis cells collected from the medium were stained with trypan blue and counted in a hemocytometer, their number being expressed as a percentage of the cell number attached to the substrate. All detached cells were found to be trypan blue positive. The medium of control cultures contained 1.5 ± 0.9% trypan blue-positive cells on day 8 and 2 ± 1% on day 10. In the medium of OH-TAM-treated cultures 5.2 ± 2.6% (day 8) and 7.5 ± 5.3% (day 10), in that of ICI 164384-treated cultures 3.9 ± 3.3% (day 8) and 5.8 ± 3.0% (day 10) trypan blue-positive cells were found.

**Kinetics of the different types of cell death**

To determine quantitatively the appearance and kinetics of cell death, cultures were fixed and stained with Hoechst fluorescent dye H-33258. Cells were categorized mainly by nuclear morphology as follows: (i) nuclei in vital cells being large with clearly visible nucleoli (Figure 5a); (ii) pyknotic nuclei (Figure 5b); (iii) nuclei with crescent chromatin (Figure 5c); (iv) fragmented nuclei (Figure 5d). In controls, nuclei with crescent chromatin and fragmented nuclei remained at a very low level (0.1–0.2%) throughout the experiment (Figure 6a). Of the cells, 1.5% exhibited a pyknotic nucleus between days 2 and 8, followed by an increase to 5% on day 10. TAM or OH-TAM at 10⁻⁶ M did not significantly increase any nuclear alteration (not shown). TAM or OH-TAM at 10⁻⁴ and 10⁻³ M showed a significant increase in the number of pyknotic nuclei.

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**Table III. Induction of cell death by 10⁻⁶ M OH-TAM treatment on day 4**

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Pyknotic nuclei</th>
<th>Nuclei with crescent chromatin</th>
<th>Fragmented nuclei</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>Co</td>
<td>1.4 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Co</td>
<td>1.3 ± 0.7</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>6</td>
<td>Co</td>
<td>1.3 ± 0.7</td>
<td>0.1 ± 0.1</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>Co</td>
<td>1.4 ± 0.8</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
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<td>8</td>
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<td>0.8 ± 0.4</td>
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<tr>
<td>9</td>
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<tr>
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<td>1.9 ± 0.6⁴</td>
<td>3.9 ± 0.3⁴</td>
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</table>

Co, control. *P < 0.01, OH-TAM versus control. Numbers, nuclear changes as a percentage (± SD) of intact cells.

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**Fig. 5.** Nuclear changes in MCF-7 cells demonstrated by H-33258 staining under the fluorescence microscope. (a) Controls. (b–d) TAM 10⁻⁶ M. (b) Pyknotic nuclei being condensed to a single mass (→). (c) Crescent pieces of chromatin at the nuclear envelope (→). (d) Fragmented nucleus with the chromatin broken down into several condensed pieces of different sizes (→).
significantly enhanced the number of nuclei with crescent chromatin and of fragmented nuclei to 4-6% between days 7 and 10 (Figure 6b, c, e and f). These nuclear alterations increased more or less in parallel. Cells exhibiting a pyknotic nucleus appeared with different kinetics and rates. After 10^{-6} M TAM or OH-TAM their incidence was increased significantly above controls within 2-3 days; at maximum ~20-25% of the cells showed this sign of death (Figure 6b, c, e and f). Thus, pyknosis predominated in cell death in MCF-7 cultures.

ICI 164 384 produced a similar pattern of nuclear signs of cell death as TAM and OH-TAM (Figure 6h and i). At the three doses tested it did not show greater potency than TAM and OH-TAM, in remarkable contrast to its effect on DNA synthesis (Table II).

When OH-TAM was added to the cultures on day 4 instead of day 1, both 10^{-7} and 10^{-6} M induced nuclear condensation and fragmentation after a lag period of ~4 days, followed by a steep increase until termination of the experiment on day 10 (Table III, data with 10^{-7} M not shown).

LDH release and trypan blue exclusion

In control cultures, no significant release of LDH into the culture medium was found until day 8, followed by a slight increase until day 10 (Figure 6d). After 10^{-6} M TAM or OH-TAM LDH activity in the culture medium increased within 1 day to almost 100% (Figure 6d and g); none of the cells excluded trypan blue (data not shown). After 10^{-7} and 10^{-6} M TAM or OH-TAM and ICI 164 384 the LDH activity in the medium did not increase before 8 days after treatment and then increased to ~15% (Figure 6d, g and j). It should be noted that the morphological changes preceded the liberation of LDH. Likewise, the number of trypan blue-positive cells in the monolayer did not increase before 7-8 days after treatment; at maximum 2-3% of the cells were stained. Similar results were obtained with 10^{-7}-10^{-5} M ICI 164 384 (data not shown).

Test for DNA degradation in MCF-7 cells after AE treatment

Chromatin fragmentation into both large (>50 kb) and subsequently into internucleosomal fragments (in some cell types) is an early characteristic of apoptotic ACD (37-39). The presence of DNA strand breaks in pyknotic nuclei such as those shown in Figure 6c was evident when the TUNEL technique was used to analyze the TAM-treated cells (data not shown). Furthermore, time course studies by PFGE revealed a clearly detectable DNA fragmentation to 50 kb from day 3 after 10^{-6} M TAM (Figure 7a). CAGE showed further DNA degradation into oligonucleosomes which, however, did not become detectable before day 3 after treatment (Figure 7a).

Nucleosomal DNA fragments of 20 kb and less also occur in the supernatant after centrifugation of cellular extracts at
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The results of PFGE and CAGE analysis of TAM-treated cultures were supported by further experiments with OH-TAM or ICI 164 384, in which the DNA content of the 27 000 g supernatant was quantitatively analyzed and which revealed no significant increase above the zero point control until 7-8 days after AE treatment (Figure 7c and d).

Furthermore, PFGE and CAGE showed that the lytic cell death occurring within 1 day after 10⁻⁵ M TAM is also associated with DNA fragmentation into 50 kb and oligonucleosomal fragments (Figure 7b).

Estrogen rescue from different types of cell death

To test for an active nature of the different types of cell death as described above, E₂ (10⁻⁹ M) was added along with or after AEs and the kinetics of nuclear changes were recorded as above (the results are plotted in Figure 8).

Formation of nuclei exhibiting crescent chromatin, fragmentation or pyknosis were all inhibited by 10⁻⁹ M E₂ (Figure 8). When E₂ was added simultaneously with AE, the number of nuclei with signs of death never exceeded control levels throughout the experiment. These findings suggest that both the apoptotic and pyknotic nuclear changes represent ACD. When E₂ was added on day 3 or 5 the number of dead cells remained approximately at the level at the time of E₂ administration, suggesting that within 3-5 days after AE treatment most of the cells were not irreversibly committed to cell death.

In contrast, E₂ at 10⁻⁹ did not prevent DNA loss after 10⁻⁵ M TAM (Figure 1). Up to 10⁻⁶ M E₂ did not protect cells from cytolysis by 10⁻⁵ M OH-TAM, as indicated by morphological examination, the trypan blue exclusion test and release of LDH into the culture medium (data not shown).

Inhibition of cell death by 3-methyladenine

3-Methyladenine has been shown to inhibit autophagy in hepatocytes by preventing the formation of autophagosomes (30). Therefore, we checked whether 3-methyladenine would affect autophagic death of MCF-7 cells after AE treatment. As shown in Figure 9, 3-methyladenine indeed caused an inhibition of TAM-induced cell death 1 and 2 days after addition. Both types of ACD were affected, the type with nuclear pyknosis to a significant extent on both days of testing. 3-Methyladenine alone did not affect the rate of cell death.

Discussion

This study demonstrates the occurrence of different types of cell death in MCF-7 cells following AE addition to the culture medium. At high concentrations (10⁻⁵ M) of TAM or its metabolite OH-TAM all cells lysed rapidly (within 24 h), as indicated by morphology at the light and electron microscopical levels, by the trypan blue exclusion test and by release of cytoplasmic enzymes into the culture medium, similarly to previous reports (19,42,43). E₂ in concentrations up to 10⁻⁶ M failed to prevent cell death induced by 10⁻⁵ M TAM or OH-
P < 0.001; **P < 0.01 using Student's t-test after TAM. Because of their low incidence, nuclei with crescent chromatin of three representative experiments are given. 3-MA was added 3 or 4 days and fragmented nuclei were summarized. •, Control; □, 3-MA alone; Δ, TAM; ○, TAM plus 3-MA. **P < 0.001; *P < 0.01 using Student's t-test.

Fig. 8. Effect of E2 on cell death in MCF-7 cultures after 4-OH-TAM and ICI 164384. AE treatment (10⁻⁶ M) on day 1, E2 (10⁻⁷ M) either on day 1, 3 or 5 (arrows). (a, c and e) OH-TAM. (b, d and f) ICI 164384. Black symbols, AE only; open symbols, AE plus E2. Vertical bars indicate SD, smaller than symbols where not shown. Symbols for statistical significance are not given for the sake of clarity of the figure.

Fig. 9. Inhibition of cell death by 3-methyladenine. 0, day of 3-methyladenine (3-MA) treatment; 1 and 2, day after 3-MA. Means (± SD) of three representative experiments are given. 3-MA was added 3 or 4 days after TAM. Because of their low incidence, nuclei with crescent chromatin and fragmented nuclei were summarized. □, Control; △, 3-MA alone; ○, TAM; ○, TAM plus 3-MA. **P < 0.001; *P < 0.01 using Student's t-test.

TAM, suggesting that this type of cell death is not mediated via the estrogen receptor. Rather, it may be explained by cytotoxic actions of TAM involving perturbations in membrane fluidity (44), formation of reactive oxygen species (45), DNA damage by DNA adducts or chromosomal aberrations, which have been found to occur in kidney and liver (46,47).

At 10⁻⁶ M and below TAM, OH-TAM and ICI 164384 induced a second type of cell death which occurred much later and was less pronounced (~35% of cells were affected at 10 days). This type of cell death is regarded as a receptor-mediated, active cell suicide, as indicated by the inhibition (rescue) by E2 even 4 days after AE treatment, thus confirming and extending a previous report on mitogen rescue in MCF-7 cells (19–21). Mitogen rescue is considered to be an important functional feature of apoptosis and other subtypes of ACD, as observed in vivo and in vitro in many different cell types, such as adrenal cortex, neuronal cells, liver, prostate, and also in hormone-dependent tumors of kidney, prostate and mammary gland (1,3,4,6,13,48–50).

AE-induced ACD in MCF-7 cells was reported previously and was designated as apoptosis (19–21). However, detailed analysis in the present study revealed profound differences from the ‘classical’ definition of apoptosis, which is based on morphological criteria. The major morphological differences of active death in MCF-7 cells from apoptosis are as follows. (i) Electron and light microscopy provided evidence for the formation of AVs [for a detailed description see Pfeifer (51)]; their number apparently increased dramatically early in the death process. As a result of extensive breakdown of the cytoplasmic organization, residual cytoplasm appeared amorphous. It is worth noting that structures required for protein synthesis, such as polyribosomes, endoplasmic reticulum and Golgi, disappeared completely, whereas a few clusters of intact mitochondria persisted in close proximity to AVs and the nuclear membrane. (ii) A pyknotic type of chromatin condensation was observed in most dying cells. (iii) Cells exhibiting crescent (apoptotic) nuclei contained numerous AVs.

According to our observations we propose a sequence of events associated with AE-induced death of MCF-7 cells (Figure 10). All stages depicted in the figure occur while cells are still attached to the substrate; detached cells exhibit signs of lysis. It should be noted that the number of cells with a normal looking nucleus but signs of extensive autophagy exceeded the number of cells exhibiting a pyknotic or apoptotic nucleus, i.e. irreversible signs of cell death. Thus, AV formation precedes irreversible signs of cell death. The importance of AVs in the preparation of cells for death is supported by the inhibition of both types of nuclear alterations by 3-methyladenine, which to our knowledge was described for the first time. 3-Methyladenine has previously been characterized as a specific inhibitor of formation of AVs in liver cells (30). Further studies on the effects 3-methyladenine in MCF-7 cells are in progress.

While the autophagic type of ACD described here has little in common with apoptosis, it closely resembles the lysosomal/autophagic or type II cell death described previously under a variety of biological conditions, e.g. during metamorphosis of the labial gland of moths (Menduca sexta), during mammalian embryogenesis, in epithelial plates after palatal closure, in the mesonephros and in adult rats during involution of the mammary gland post-weaning (24,25). It therefore appears that the autophagic mode of cell death is not peculiar to MCF-7 cells, but is an important mechanism in tissue homeostasis of...
DNA degradation after a lytic dose of TAM is much more rapid than during ACD. Most probably membrane destruction results in an increase in cytoplasmic and nuclear Ca\(^{2+}\) and Mg\(^{2+}\) ions, thereby activating DNases (37,53). Likewise, necrosis of neuronal cells has been found to be associated with transient formation of oligonucleosomes (54). These observations emphasize current notions that certain patterns of DNA fragmentation (e.g. DNA ladders) should not be considered specific for the apoptotic mode of ACD (6,52,54).

Our study confirms the well-known inhibition of DNA synthesis and mitosis of MCF-7 cells by AEs (14,16,17,55). However, AEs obviously have an additional effect on these cells, namely induction of cell death or anti-survival activity. Our results suggest that the anti-proliferative and anti-survival actions may, to some extent, be independent of each other: (i) the inhibition of survival becomes manifest several days later than the anti-proliferative effect and can be reversed by estrogen at day 4 when replication in uninhibited cells is at its peak (and therefore not susceptible to AE); (ii) the lowest TAM concentration tested (10\(^{-8}\) M) exerted an anti-proliferative effect, but no anti-survival effect; (iii) E\(_2\) reversed the anti-survival effect of ICI 164384, but not its anti-proliferative effect; (iv) ICI 164384 and TAM differed ~10-fold in anti-survival potency, but their anti-survival effect was equal. Likewise, studies in other tissues, such as liver (6), oligodendrocytes and chondrocytes (5), have shown remarkable differences in the potency of growth signals to promote survival or proliferation. The obvious separation between regulation of proliferation and of survival in MCF-7 cells and the availability of a variety of well-defined estrogen agonists and antagonists may provide a useful experimental model to study the underlying molecular events. As to cancer treatment, use of AEs with high anti-survival activity hypothetically promises particular therapeutic efficacy. Our study provides a rationale and means of selecting compounds suitable for further exploration of this hypothesis in pre-clinical and clinical testing.

A further point of interest is the question of which factor(s) in our culture system stimulate proliferation and survival of MCF-7 cells. It was expected that charcoal treatment removes

![Hypothetical diagram of stages of death of cultured MCF-7 cells after TAM. Autophagy, formation of AVs. Chromatin condensation, condensation of heterochromatin and detachment from nuclear envelope (the latter remains intact) with few polyribosomes. Pyknosis, condensation of the chromatin to a single mass in the center of the nucleus (still intact nuclear envelope) with amorphous cytoplasm with few clusters of AVs and mitochondria. In cell culture, the cells finally detach from the substrate and undergo secondary necrosis (not included).]
most or all estrogens and estrogen-like compounds that may be present in FCS. Nevertheless, in our study, as well as in others (14,16,17), AEs were clearly inhibitory, suggesting involvement of the estrogen receptor. Several explanations for these surprising observations can be envisaged. (i) Charcoal stripping may not remove all estrogenic compounds from the serum. (ii) The stimulating factors in the serum may be peptide growth factors such as EGF or IGF-I, which have been found to be mitogenic for breast cancer cells; the apparent involvement of the estrogen receptor might occur via receptor cross-talk (11,17,56). A recent study with MCF-7 and T47D cells revealed that TAM, in remarkable contrast to pure AEs such as RU 58668, ZM 182780 and ZK 164015, did not affect or even enhance estrogen receptor levels (57). (iii) The estrogen antagonists may act as ‘inverse agonists’ for estrogen receptors (58,59). This would imply that estrogen receptors in resting MCF-7 cells may be in equilibrium between an active and an inactive state; estrogens would favor transition into the active, AEs transition into the inactive state. The current findings may provide interesting models to tackle some of these possibilities.

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References

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