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Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments

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SUMMARY

Programmed cell death comprises several subtypes, as revealed by electron microscopy. Apoptosis or type I programmed cell death is characterized by condensation of cytoplasm and preservation of organelles, essentially without autophagic degradation. Autophagic cell death or type II programmed cell death exhibits extensive autophagic degradation of Golgi apparatus, polyribosomes and endoplasmatic reticulum, which precedes nuclear destruction. In the present study, we analysed the fate of cytokeratin and F-actin during autophagic cell death in the human mammary carcinoma cell line MCF-7 because recent studies suggest that an intact cytoskeleton is necessary for autophagocytosis. Programmed cell death was induced by 10^{-6} M tamoxifen. For quantitative light microscopic analysis, autophagic vacuoles were visualized by monodansyl cadaverin, which stains autophagic vacuoles as distinct dot-like structures. In control cultures, the number of monodansylcadaverin-positive cells did not exceed 2%. Tamoxifen induced a dramatic increase 2-4 days after treatment to a maximum of 60% monodansylcadaverin-positive cells between days 5 and 7. Cell death, as indicated by nuclear condensation, increased more gradually to about 18% of all cells on day 7. In cells with pyknotic nuclei cytokeratin appeared disassembled but retained its immunoreactivity; actin was still polymerized to filaments, as demonstrated by its reaction with phalloidin. Western blot analysis showed no significant cleavage of the monomeric cytokeratin fraction. For comparison, apoptotic or type I cell death was studied using the human colon cancer cell HT29/HI1 treated with the tyrosine kinase inhibitor tyrphostin A25 as a model. Cleavage of cytokeratin was already detectable in early morphological stages of apoptosis. F-actin was found to depolymerize; its globular form could be detected by antibodies; western blot analysis revealed no products of proteolytic cleavage. In conclusion, in our model of apoptosis, early stages are associated with depolymerization of actin and degradation of intermediate filaments. In contrast, during autophagic cell death intermediate and microfilaments are redistributed, but largely preserved, even beyond the stage of nuclear collapse. The present data support the concept that autophagic cell death is a separate entity of programmed cell death that is distinctly different from apoptosis.

Key words: MCF-7, HT29/HI1, Tamoxifen, Tyrphostin A25, Autophagic cell death, Apoptosis, Cytokeratin, Actin

INTRODUCTION

Cell death by apoptosis (often called ‘programmed cell death’, PCD) serves two major functions: (1) as an antagonist to mitosis for the controlled elimination of excessive cells that are no longer needed by the organism (e.g. during atrophy or involution of tissues); (2) as one of the organism’s defense lines against disease, e.g. to eliminate (pre)neoplastic cells or damaged cells after cytotoxic injury. The concept of apoptosis helped to elucidate the response of various tissues to toxic injury as well as mechanisms of carcinogenesis and teratogenesis. Thus, induction of apoptosis, in addition to inhibition of cell proliferation, is now a major goal of strategies for cancer prevention as well as for treatment of frank neoplasia (for reviews, see Hickman and Boyle, 1997; Roberts et al., 1997; Schulte-Hermann et al., 1995; Wyllie, 1997). In the last decade apoptosis has attracted increasing scientific interest and significant progress has been achieved in understanding its control by survival and death factors as well as the intracellular events associated with the cell’s suicide. However, accumulating morphological and biochemical evidence suggests that programmed cell death is not confined to apoptosis. The term ‘apoptosis’ was originally conceived on morphological grounds, namely describing the condensation of chromatin at the nuclear membrane, prominent condensation of cytoplasm and the fragmentation of cell and nucleus, while organelles were well preserved and autophagocytosis was essentially absent (Kerr et al., 1972). On the other hand, in numerous biological systems the cell’s suicide program has been found to involve the autophagic/lysosomal compartment.

Thus, cell death-associated autophagy has been observed in the fungus *Dictyostelium discoideum* during starvation-induced sorocarp formation (Cornillon et al., 1994). Furthermore, autophagic cell death has been observed in physiological states of development (e.g. during insect metamorphosis, mammalian embryogenesis: regression of interdigital webs, sexual anlagen) and adulthood (e.g. in intestine, mammary gland post-weaning, ovarian atretic follicles) (Beaulaton and Lockshin, 1977; Clarke, 1990; Schweichel and Merker, 1973; Shibahara et al., 1995; Zakeri et al., 1995; D'Herde et al., 1996; Jochova et al., 1997). Autophagic cell death also appears to be associated with experimental and human (Alzheimer, Parkinson) neurodegenerative diseases (Cataldo et al., 1995; Anglade et al., 1997; Migheli et al., 1997). It is important to note that autophagic cell death and apoptosis may occur in the same tissue. Recent studies on cell death in human glioma and gastric cancer cells suggest that Ras expression plays a key role in triggering either pathway of cell death: overexpression of Ras was found to result in an autophagic type of cell death without activiation of caspase-3-like protease activity, whereas TNF-α induced apoptosis in the same cell line (Chi et al., 1999). However, the pathway(s) leading to either apoptotic or autophagic cell death are at present poorly understood (for a review, see Kitanaka and Kuchino, 1999). Recently, we have described the occurrence of autophagic cell death in human mammary carcinoma cells (MCF-7) after treatment with tamoxifen: cell death as indicated by nuclear fragmentation and pyknosis was preceded by autophagic elimination of ribosomes, Golgi apparatus and endoplasmatic reticulum, and the residual cytoplasm appeared amorphous (Bursch et al., 1996). In the present study we addressed the fate of the cytoskeleton during autophagic cell death as well as in 'classical' apoptosis. Depolymerization or cleavage of actin, cytokeratins, lamins and other cytoskeletal proteins have been found to be involved in the cell's preparatory as well as executional steps of apoptosis (Bonfoco et al., 1996; Brancolini et al., 1997; Brown et al., 1997; Caulin et al., 1997; Chen et al., 1996; Kayalar et al., 1996; Kothakota et al., 1997; Kruidering et al., 1998; Ku et al., 1997; Levee at al., 1996; Porter and Jänicke, 1999; Schmeiser and Grand, 1999; Takahashi et al., 1996; Tinnemans et al., 1995; van Engeland et al., 1996, 1997). On the other hand, autophagocytosis is known to depend on the intact cytoskeleton. Thus, intermediate and microfilaments are considered to be essential for the initial formation of autophagosomes, whereas their subsequent fusion with lysosomes depends on microtubuli (for a review, see Bloomart et al., 1997). Therefore, we wondered whether cleavage of the cytoskeleton as described for apoptosis would occur during autophagic cell death. We initiated a comparative study on cytokeratin and actin in two experimental models, namely autophagic cell death in MCF-7 cells induced by tamoxifen as well as apoptosis in colon cancer cells (HT29/HI1) after tyrphostin A25. The results of immuno- and histochemical as well as western blot analysis revealed that apoptotic cell death of colon cancer cells is characterized by breakdown of cytokeratin and depolymerization of F-actin. In contrast, during autophagic cell death of MCF-7 cells, cytokeratin disassembled but was not degraded to a significant extent until detachment of the dead cells. Furthermore, F-actin was preserved even in cells with pyknotic nuclei, i.e. in the execution stage of programmed cell death. Thus, cytoskeletal proteins appear at least partially preserved during autophagic-driven cell death. This pattern of changes is clearly different from that ascribed to apoptosis. Human breast cancer cells such as MCF-7 are routinely used to evaluate the anti-tumor potency of anti-estrogens and other drugs (Bardon et al., 1987; Detre et al., 1999; Johnston et al., 1997; Jordan et al., 1990; Osborne et al., 1995; Otto et al., 1996; Wilson et al., 1995). Thus, better understanding of the cell's suicide mechanisms may eventually help to elucidate new targets for therapeutical intervention.

MATERIALS AND METHODS

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): Triton-X-100, NADH (disodium salt), insulin and Hepes. EDTA, sodium dodecyl sulfate, Tris, Geltol and Entellan were from Merck (Darmstadt, Germany); H-33258 was from Riedel-De-Haen (Hannover, Germany), tamoxifen was purchased from Sigma.

MCF-7 human breast cancer cells were kindly provided by Dr A. M. Otto, University of Regensburg, Germany. Culture conditions were described in detail previously (Bursch et al., 1996). Briefly, cells were grown as a monolayer in DMEM without Phenol Red supplemented with 10% fetal calf serum (FCS), L-glutamine (300 mg/l), bovine insulin (100 U/ml) and Hepes (10 mM) at 37°C in an atmosphere of 5% CO_2. Cells were detached from the substrate using trypsin/EDTA (0.05:0.02%) and further separated by the application of a syringe containing a 22-gauge needle. 7 days before the beginning of an experiment cells were steroid-withdrawn according to Bardon et al. (1987). Cells were plated on glass coverslips in plastic dishes (diameter 35 mm) at a density of $7.5\times10^3/cm^2$ and the culture medium was replaced by DMEM containing 3% charcoal-stripped-FCS. 24 hours later the cells were treated with tamoxifen ([α-(4-β-*N*-dimethylaminoethoxy)-phenyl-α′-ethyl-*trans*-stilbene]; TAM). For treatment, 2 μl of freshly prepared dilutions of the test substances in DMSO/ethanol (1:1, v:v) were added directly to the medium. Controls were treated with DMSO/ethanol (0.5%).

HT29/HI1 colon adenocarcinoma cells were a gift from Dr E. Friedman, Memorial Sloan-Kettering Cancer Center, New York, NY, USA. They were kept under standard tissue culture conditions using DMEM containing 10% FCS (Hafez et al., 1990). For the induction of apoptosis, cells were treated with 16 μg/ml tyrphostin A25 (Sigma) dissolved in DMSO (final concentration 0.5%).

Morphological and histochemical procedures

Nuclear morphology

Whole-mount cells attached to glass coverslips were rinsed twice with PBS, fixed with ice-cold paraformaldehyde (3% in PBS) for 10 minutes and washed twice with distilled water. After drying cells were stained for 10 minutes with a freshly prepared solution of H-33258 in phosphate-buffered saline (PBS; 3 mg/ml, pH 5) and incubated for 5 minutes in citrate buffer (0.1 M sodium citrate, pH 3.0) in the dark. Thereafter cells were washed once with distilled water and once with ethanol, and the glass coverslips were mounted onto slides using Mowiol as a mounting medium.

Electron microscopy

The 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 minutes. After washing in PBS the cells were postfixed in OsO_4 and embedded in Epon; 0.1 μm thin sections were stained with uranyl acetate/lead citrate (Fluka) and viewed in a Philips EM 400 electron microscope.

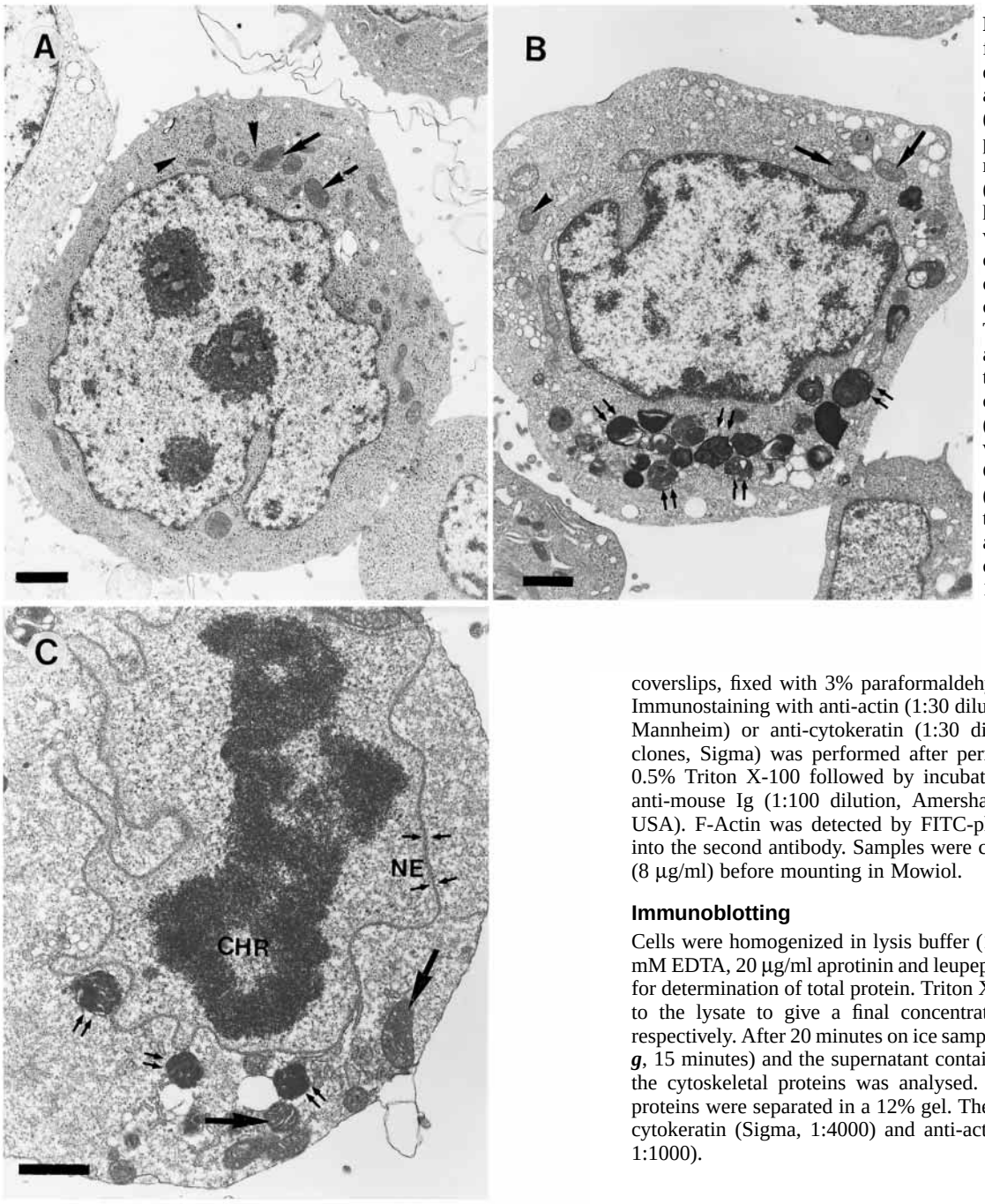


Fig. 1. Ultrastructural features of autophagic cell death in MCF-7 cultures after TAM treatment. (A) Control, 168 hours; polyribosomes (arrowhead), mitochondria (arrow). (B) 10^{-6} M TAM, 168 hours, numerous autophagic vacuoles (double arrows), chromatin appears slightly condensed as compared to control (cf. A). (C) 10^{-6} M TAM, 168 hours, amorphous appearance of the cytoplasm, containing clusters of mitochondria (arrow) and autophagic vacuoles (double arrows). Condensed chromatin (CHR) has detached from the nuclear envelope (NE) and concentrated in the center of the nucleus. Bars, 1 μm.

Autophagic vacuoles

Autophagic vacuoles (AV) were detected with monodansylcadaverin (MDC) according to the method of Biederbick et al. (1995). Cultures were incubated with 0.05 mM MDC for 60 minutes at 37°C followed by fixation in 4% paraformaldehyde (15 minutes), washed twice with PBS and counterstained with propidium iodide (5 minutes 12.5 μg/ml in PBS). The glass coverslips were mounted onto slides using Geltol as mounting medium. Quantitative determination of MDC-positive cells was performed immediately after preparation, because the MDC-fluorescence bleaches quickly, using a Nikon Mikrophot-FXA equipped with a 356 nm excitation filter and a 545 nm barrier filter.

Immunocytochemistry

For the detection of cytoskeletal proteins cells were grown on glass coverslips, fixed with 3% paraformaldehyde and washed with PBS. Immunostaining with anti-actin (1:30 dilution, clone C4, Boehringer-Mannheim) or anti-cytokeratin (1:30 dilution, C2562, mixture of clones, Sigma) was performed after permeabilization of cells with 0.5% Triton X-100 followed by incubation with Texas Red-linked anti-mouse Ig (1:100 dilution, Amersham, Arlington Heights, IL, USA). F-Actin was detected by FITC-phalloidin diluted (2 μg/ml) into the second antibody. Samples were counterstained with H33258 (8 μg/ml) before mounting in Mowiol.

Immunoblotting

Cells were homogenized in lysis buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 20 μg/ml aprotinin and leupeptin) and a sample was taken for determination of total protein. Triton X-100 and NaCl were added to the lysate to give a final concentration of 1% and 500 mM, respectively. After 20 minutes on ice samples were centrifuged (30000 ***g***, 15 minutes) and the supernatant containing the soluble fraction of the cytoskeletal proteins was analysed. Constant amounts of total proteins were separated in a 12% gel. The antibodies used were anti-cytokeratin (Sigma, 1:4000) and anti-actin (Boehringer Mannheim, 1:1000).

RESULTS

Autophagic cell death in MCF-7 cells induced by Tamoxifen

We have previously shown the occurrence of autophagic vacuoles (AVs) in TAM-treated MCF-7 cells by electron microscopy and in semithin sections stained with Toluidine Blue; typical electron microscopical features of TAM-induced autophagic cell death are shown in Fig. 1 (for details see Bursch et al., 1996). We now report that monodansylcadaverin (MDC) is taken up by MCF-7 cells from the culture medium and is selectively accumulated in AVs; when viewed by fluorescence microscopy AVs appear as distinct dot-like structures (Fig.

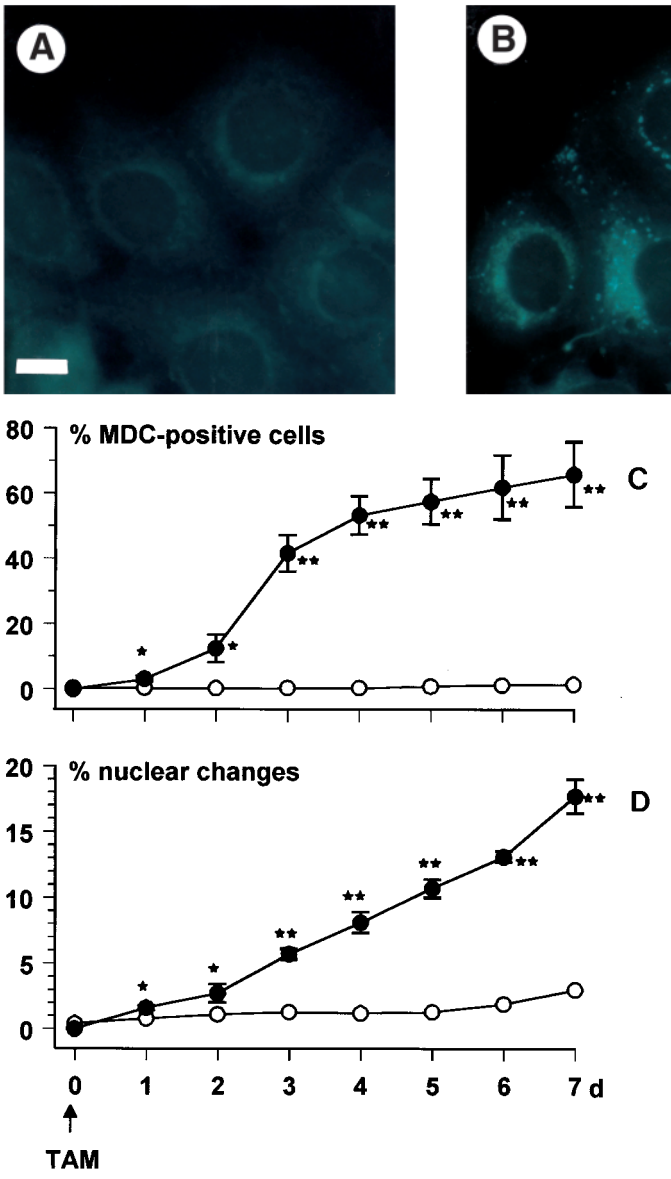


Fig. 2. Detection of autophagic vacuoles in MCF-7 cells by monodansylcadaverin (MDC; see Materials and Methods for details). (A) Control, (B)10^{-6} M tamoxifen, day 5; AVs (arrow); bar, 4 μm. (C) Percentage of MCF-7 cells with autophagic vacuoles (designated MDC-positive cells). (D) Percentage of cells with nuclear changes indicative of cell death: for quantitative analysis vital and dead cells were classified according to their nuclear structure. Vital cells: large, round nuclei with visible nucleoli. Dead cells: (1) pyknotic nuclei, (2) nuclei with crescent chromatin, (3) fragmented nuclei (see Bursch et al., 1996 for detailed morphological description). (○) control, (●) 10^{-6} M tamoxifen. In C and D, 1000 cells in each of triplicate cultures were counted; the total number of MDC-positive cells as well as of dead cells (sum of all types of nuclear alterations) are expressed as percentage of total cells scored. Means of three experiments are shown. Vertical bars indicate s.d.; when not shown, they are smaller than the symbols.*P<0.05; **P<0.01, control versus TAM using Student's t-test.

2A,B). In control cultures, only very few cells contained AVs (designated MDC-positive cells) and their incidence did not change during the entire period of the experiment (Fig. 2C). After TAM treatment, both the number of AVs per cell and the percentage of MDC-positive cells increased dramatically between 2 to 4 days and leveled off at about 60% between day 5-7 (Fig. 2C). Nuclear condensation was determined as an indicator of cell death, and it exhibited a more gradual increase to about 18% on day 7 (Fig. 2D). This is in accordance with our previous conclusions that autophagocytosis precedes cell death in MCF-7 cells.

In further experiments cytokeratin and F-actin were visualized in individual MCF-7 cells by immuno- and histochemical means. In intact cells, cytokeratin and F-actin form fine-meshed networks (Fig. 3B,D). In dead cells, as indicated by pyknotic or fragmented nuclei, the following features were found: (1) cytokeratin appears disassembled into dot-like structures (Fig. 3A,B); (2) actin was still found to be polymerized to filaments as demonstrated by its interaction with phalloidin; the filaments aggregated around the nucleus with star-like extensions to the cell membrane (Fig. 3C,D). Quantitative analysis revealed that the vast majority of MCF-7 cells exhibiting either pyknotic or fragmented nuclei still contained F-actin, as demonstrated by phalloidin-staining (Table 1). Western blot analysis of cytokeratin and actin was performed. Controls exhibited the typical cytokeratin pattern of MCF-7 cells with CK 8 (52 kDa), CK 18 (48 kDa) and CK 19 (40 kDa). An additional band appeared at 33 kDa in both controls and TAM-treated cells (Fig. 3E). TAM-treatment did not result in significant changes of this pattern until day 7. However, a weak band running between CK18 and CK19 appeared on day 3 with a subsequent increase in intensity on day 7 (Fig. 3E). A more pronounced fragmentation of cytokeratin was found in dead cells detached from the substrate (data not shown). Western blot analysis of total actin revealed a slight reduction in intensity with time in controls. In TAM-treated cultures, a slight reduction in the intensity of the bands could be detected on day 7 but not before. These observations show that the cytoskeletal proteins do not undergo significant degradation during TAM-induced PCD in MCF-7 cells, at least not before detachment from substratum.

Apoptosis in HT29/HI1 colon cancer cell line induced by tyrphostin A25

The type of cell death induced in HT29/HI1 cells by tyrphostin was examined by electron microscopy. Control cells were of polygonal shape and attached to each other; microvilli were visible in those surface areas exhibiting no cell-cell contact. The cytoplasm contained polyribosomes, endoplasmatic reticulum and a few autophagic vacuoles (Fig. 4A). Tyrphostin-treated cells exhibited typical signs of apoptosis as shown in Fig. 4B, including rounded cells with loss of microvilli, separated from each other, and condensation of the chromatin to crescent pieces that abut with the nuclear membrane. The mitochondrial structure appeared well preserved and polyribosomes were still visible, but the Golgi apparatus appeared dilated. Some autophagic vacuoles were seen but their number was not elevated above controls (Fig. 4B). The latter observation was confirmed by applying the MDC technique (data not shown). As to the cytoskeleton, the cytokeratin of intact cells appeared as a fine-meshed network (Fig. 4D). Cells with fragmented nuclei, however, exhibited only a very weak immunoreactivity

against anti-cytokeratin (Fig. 4C,D). Likewise, the vast majority of cells exhibiting pyknotic and fragmented nuclei did not contain F-actin as revealed by a negative phalloidin staining (Fig. 4E,F, Table 1). These cells, however, retained immunoreactivity against anti-actin, thus demonstrating the presence of actin in its globular form (Fig. 4E-G). These observations were verified by western blot analysis. 24 hours after tyrphostin treatment the cytokeratin of adherent cells showed weak bands below 30 kDa (Fig. 4H). Detached apoptotic cells collected from the medium over a 1 or 24 hour period showed more fragments and an increased intensity of all bands. Western blot analysis of total actin, however, revealed no immunodetectable degradation products (Fig. 4H).

DISCUSSION

In the present study we used two experimental model systems to compare changes that develop in the cytoskeleton during the apoptotic and the autophagic mode of cell death. Autophagic cell death was induced in human mammary carcinoma cells MCF-7 by tamoxifen. We show that the MDC procedure previously described by Biederbick et al. (1995) can be used to visualize the occurrence of AVs in MCF-7 cells. Assaying MDC-positive dots per cell and MDC-positive cells per population gives a quantitative assessment of the role of AV in autophagic cell death. Numbers of MDC-positive cells steeply increased between 2 and 4 days after TAM treatment reaching a maximal incidence of about 60%. Nuclear pyknosis and fragmentation, i.e. the irreversible stage of cell death, increased later and to a lesser extent (18% on day 7). These findings provide quantitative support for previous electron-microscopical data showing that the number of cells exhibiting a normal-appearing nucleus in the presence of extensive autophagy exceeds the number of those cells exhibiting a pyknotic nucleus. Obviously, AV formation preceded the

Table 1. Percentage of dead cells containing F-actin as demonstrated by phalloidin staining

	Cells staining with phalloidin*	
Cells and treatment	Positive	Negative
MCF-7 cells		
Co, d1	0	1
Co, d7	54	0
TAM, d1	10	6
TAM, d7	147	31
Total	211	38
Percentage	85% (79,6-88,9)	15% (11-20,3)
HT29/HI1 cells		
Co, d2	1	38
TYR, d2	5	87
Total	6	125
Percentage	5% (1,6-9,7)	95% (90,2-98,3)

Induction of cell death and double staining with phalloidin and H33258. For details, see Materials and Methods.

*In two experiments cultures were scored for cells with pyknotic or fragmented nuclei exhibiting either positive or negative staining with phalloidin.

Co, control; TAM, tamoxifen; TYR, tyrphostin A 25; d1, 2 and 7, day 1, 2 and 7 of culture.

Numbers of cells found in control and treated cultures are given, as well as the percentage of the total number of dead cells. 95% confidence limits are given in parentheses.

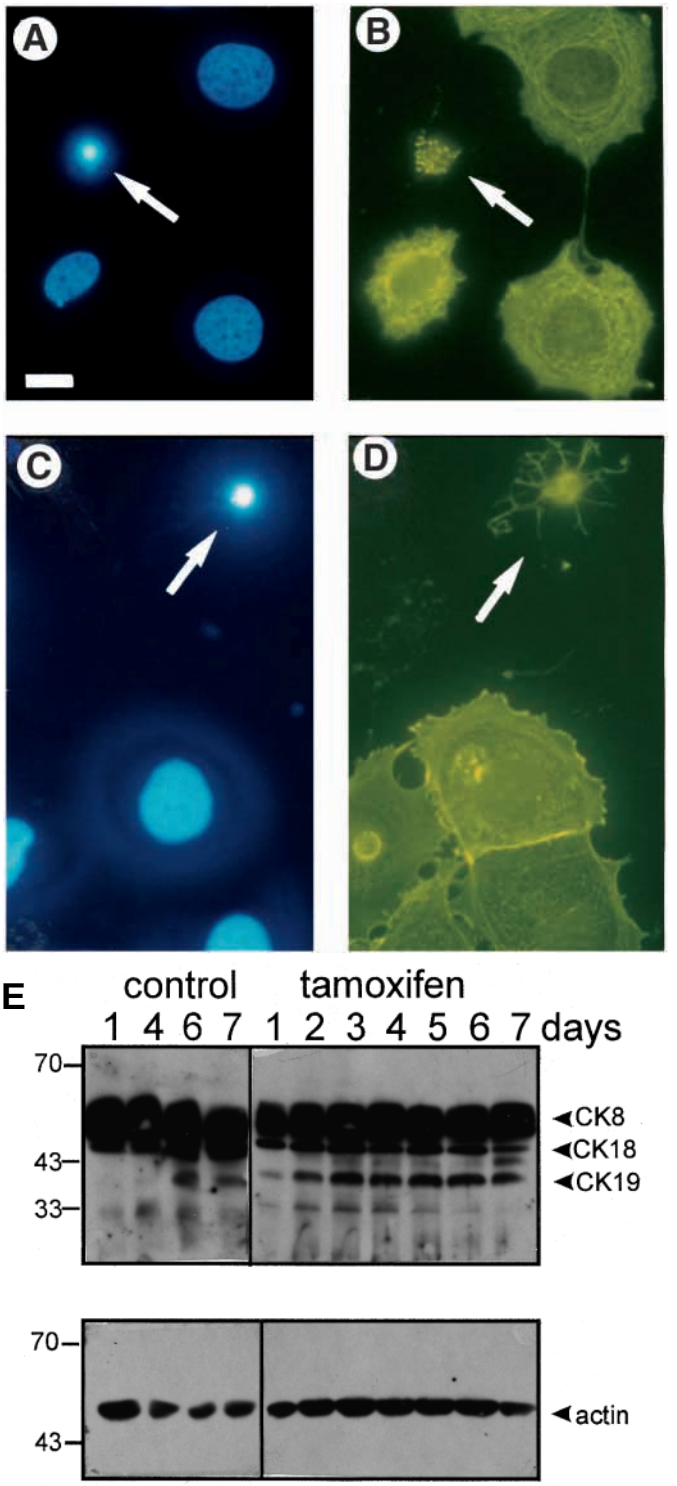


Fig. 3. Cytokeratin and actin in MCF-7 cells before and after tamoxifen treatment. (A-D) Immuno- and histochemical demonstration. (A,C) H33258 to demonstrate the nuclear structure. (B) Cytokeratin, (D) F-actin; 7 days after 10^{-6} M tamoxifen. Arrows indicate cells with a pyknotic nucleus (A,C) and demonstration of cytokeratin (B, dot-like structure) or F-actin (D, star-like extensions to plasma membrane). Bar, 4 μm. (E) Western blot analysis of cytokeratin and total actin in MCF-7 cells. Control: 1,4,6,7 days; Tamoxifen (10^{-6} M): 1-7 days after treatment. Molecular mass (kDa) is indicated at the left; CK, cytokeratin. A representative blot of four experiments is shown.

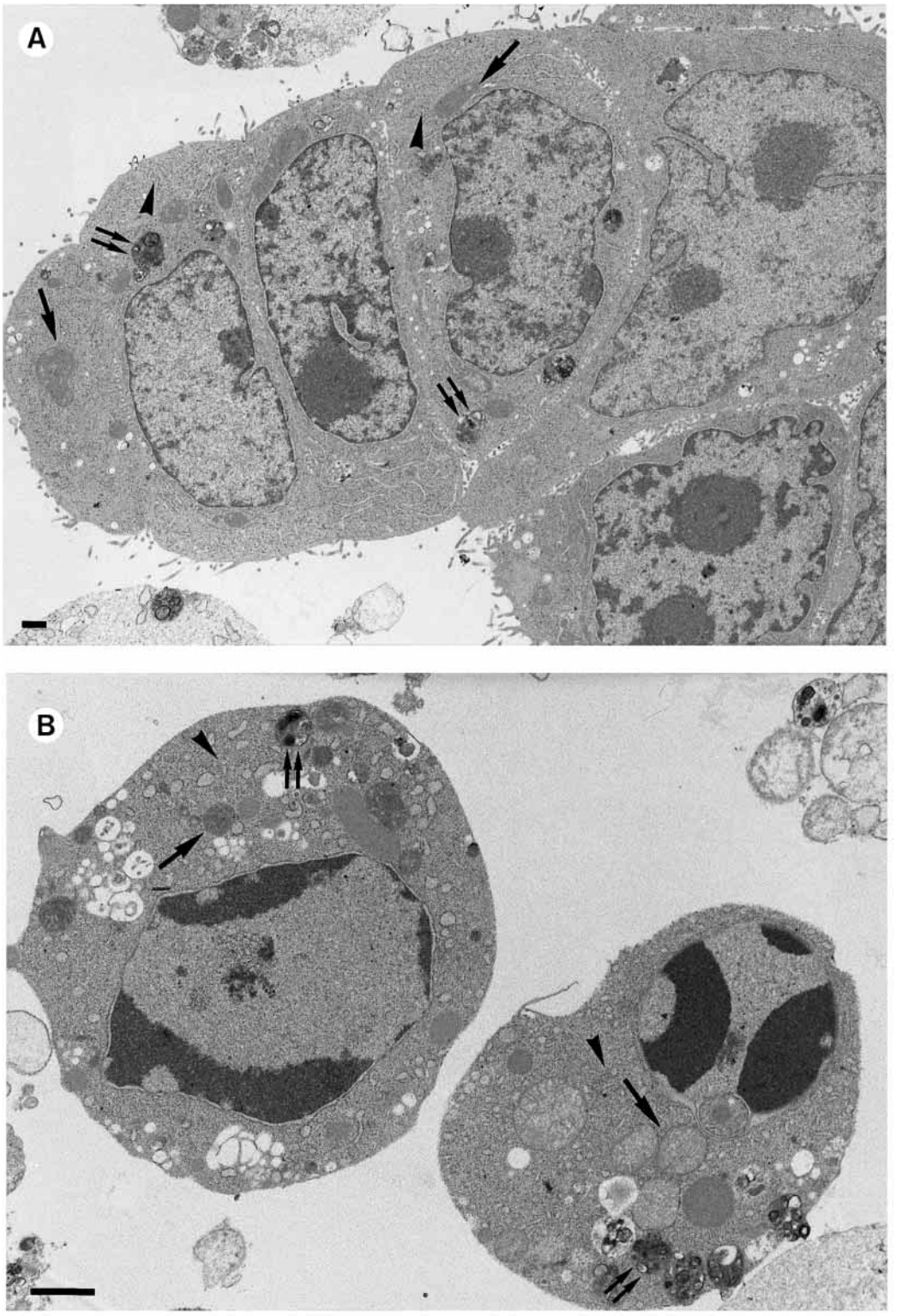
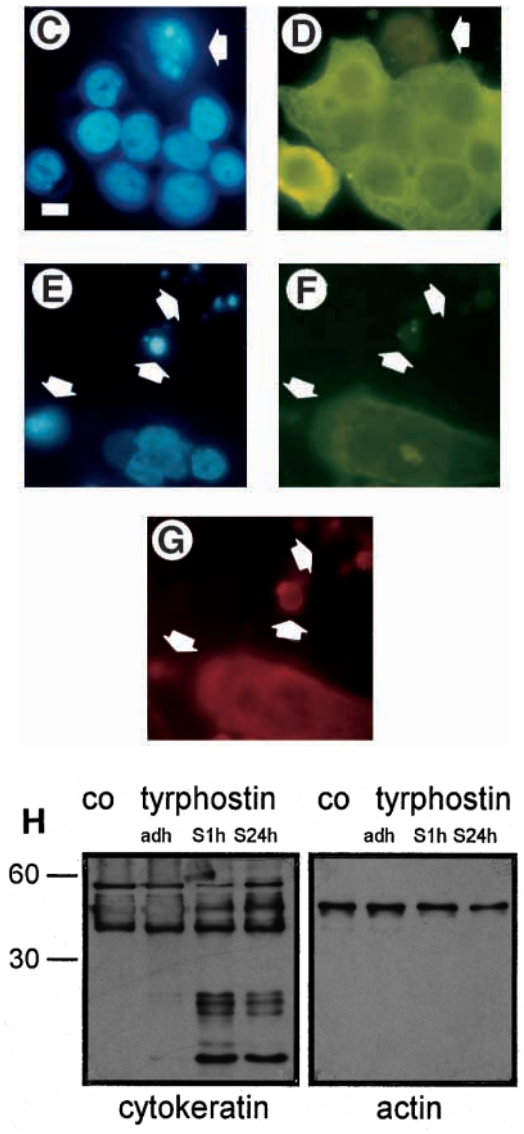


Fig. 4. Morphology, cytokeratin and actin in HT29/HI1 cells before and after treatment with tyrphostin A25. Electron-microscopical features: (A) control; (B) apoptotic cells 24 hours after 16 μg/ml tyrphostin A25; polyribosomes (arrowhead), mitochondria (arrow); mitochondra in left cell show slightly dilated cristae and electron dense matrix, and in the right cell, narrow cristae, electron lucent matrix), autophagic vacuoles (double arrows); bars in A and B, 1 μm. (C-G) Light microscopical features 24 hours after treatment with tyrphostin A 25 (16 μg/ml). (C,E) H33258 to demonstrate the nuclear structure; (D) cytokeratin, note only very weak immunoreactivity around fragmented nucleus (arrow); (F) F-actin, note very weak or lack of phalloidin staining around fragmented nuclei (arrows); (G) actin, as demonstrated by immunoreactivity against an antibody recognizing a highly conserved epitope present in all six mammalian actins (Boehringer clone C4) (arrows). Bar, 4 μm. (H) Western blot analysis of cytokeratin (left panel) and actin (right panel). Co, control; adh, adherent cells; S1h, S24h: cells collected from the medium within 23-24 hours (S1h)or 0-24 hours (S24h) after treatment. A representative blot of three experiments is shown.

execution stage of cell death, as shown before (Bursch et al., 1996). In these studies we found that cell death, as indicated by nuclear condensation, further increased beyond day 7 but cells should not be cultured longer than day 10 because then general degenerative changes occurred in TAM-treated as well as in control cultures (Bursch et al., 1996). Thus, the seemingly inconsistent number of MDC-positive cells and those cells exhibiting nuclear condensation 7 days after TAM most probably reflects differences between the kinetics of initiation and completion of cell death. The importance of autophagic vacuoles in the preparation of cells for death was supported by functional criteria. Thus, nuclear condensation was inhibited by 3-methyladenine (3-MA; Bursch et al., 1996). 3-MA has been characterized as a specific inhibitor of formation of autophagic vacuoles in liver cells and recently we found that 3-MA also inhibits the formation of AVs in MCF-7



cells (Seglen and Gordon, 1982; L. Török and W. Bursch, unpublished observation). Formation of autophagic vacuoles preceding the nuclear manifestation of cell death has been also observed in leukemic and glioblastoma cells; overexpression of Ras served as the death signal (Chi et al., 1999; Jia et al., 1997). As in our studies with MCF-7 cells, 3-MA inhibits both, the formation autophagic vacuoles and the progression to death of leukemic and glioblastoma cells (Chi et al., 1999; Jia et al., 1997).

Recent concepts suggest that the cytoskeleton is necessary for autophagocytosis (for a review, see Bloomaart et al., 1997). Sequestration of cytoplasmic structures, the initial step in formation of autophagosomes, depends on intermediate filaments such as cytokeratin and vimentin (Aplin et al., 1992; Blankson et al., 1995). Furthermore, all steps including the degradation of cytoplasmic material in AVs are ATP-dependent (Luiken et al., 1996; Plomp et al., 1988, 1989; Schellens and Meijer, 1991). Our present observations on autophagic-driven cell death match with this concept. Thus, even MCF-7 cells with pyknotic nuclei, i.e. in the irreversible stage of cell death, retained immunoreactivity against anti-cytokeratin, even though the intermediate filaments exhibited a dot-like distribution pattern. These structures most probably are associated with mitochondria and AVs clustered at the cell poles, as previously observed by electron microscopy (Bursch et al., 1996). Western-blot analysis of adherent cells suggested that most of the cytokeratin remained undegraded until detachment, except for some cleavage as indicated by the occurrence of a weak band running between CK18 and CK19. This band was not detected before day 3-4, i.e. when the number of MDC-positive cells had already reached its maximum. Furthermore, a more pronounced fragmentation of the cytokeratin could be detected in MCF-7 cells detached from the substrate and therefore probably in a stage of secondary necrosis. Taken together, the present findings suggest that the cytokeratin is redistributed but not degraded to a significant extent and probably functionally intact in the course of autophagic degradation and cell death until late stages. Furthermore, actin was still found to be polymerized to F-actin even in the vast majority of cells with pyknotic nuclei. Western blot analysis of total actin revealed no signs of proteolytic cleavage; this finding has been confirmed by the two-dimensional electrophoresis technique (Ch. Gerner and W. Bursch, unpublished observation). Moreover, polymerization of G- to F-actin is an ATP-dependent process and, therefore, F-actin is a sensitive indicator of the metabolic state of a cell. These observations are supported by previous studies showing that at late stages of the death process, when most of the cytoplasm appeared amorphous, the few remaining AVs were associated with clusters of structurally and functionally intact mitochondria as shown by electron microscopy and rhodamine 123 staining. The preservation of mitochondria and thus ATP synthesis throughout autophagic degradation was also supported by the observation that mitochondrial dehydrogenase activity did not decrease with time in TAM-treated MCF-7 cultures (L. Török and W. Bursch, unpublished observation). Hypothetically, ATP synthesis is required during type II programmed cell death while extensive autophagic degradation of polyribosomes and Golgi apparatus occurs.

In the present study we also analysed the fate of cytoskeletal components during ‘classical’ apoptosis. In colon cancer cells (HT29/HI1) the tyrosine kinase inhibitor tyrphostin A25 was found to induce apoptosis, as shown unequivocally by electron microscopy. A few AVs seen in apoptotic cells also occurred in vital control cells with similar frequency; these AVs are considered to reflect normal protein turnover such as high-mannose glycoproteins and sphingo(glyco)lipids (Ghidoni et al., 1996; Houri et al., 1993). Adherent apoptotic cells exhibiting fragmented nuclei showed only very weak or no immunoreactivity against anti-cytokeratin. Western blot analysis of cells attached to the substrate revealed some cleavage of the monomeric cytokeratin fraction below 30 kDa. However, detached apoptotic cells collected from the medium over 1 or 24 hours showed a clear-cut degradation of cytokeratin. This may be explained by the rapid detachment of HT29/HI1 cells once they reach the stage of chromatin condensation and fragmentation. Thus, at any given time point only a relatively small portion (about 2%) of cells with apoptotic nuclear morphology are still integrated into the monolayer, in spite of a total cell loss of about 60% within 48 hours after tyrphostin (Partik et al., 2000). In summary, intermediate filaments are subjected to substantial degradation

during apoptosis, which appears to start before detachment of apoptotic cells from the substrate and fragmentation into apoptotic bodies and/or secondary necrosis. Similar observations have been made in studies on olomoucine-, roscovitin- or cycloheximide-induced apoptosis of human non-small cell lung cancer and neuroblastoma cells (Tinnemans et al., 1995; van Engeland et al., 1996, 1997). Thus, the first signs of aggregation of cytokeratin and vimentin were found to coincide with the exposure of phosphatidylserine on the outer cell membrane. Further downstream in the apoptotic process the intermediate filaments appear to be proteolytically cleaved and, subsequently, the apoptotic cells detach from the substrate. Likewise, Ku et al. (1997) reported, using epitope-defined keratin antibodies, specific proteolytic cleavage of human type I keratin 18 into 28 and 20 kDa, as well as of keratin 19 into 29 and 23 kDa fragments, during anisomycin-induced apoptosis of HT29 cells.

A further result of our studies was that F-actin is depolymerized to G-actin in more than 90% of the HT29/HI1 cells exhibiting an apoptotic nucleus but not yet detached from the substrate, i.e. an early stage of apoptosis in HT29/H11 cells. Western blot analysis revealed no signs of proteolytic cleavage of total actin. Likewise, Guénal et al. (1997) observed only very little proteolysis of actin during early stages of apoptosis in rat embryo fibroblasts (REtsAF). Rather, non-polymerized actin accumulated in apoptotic bodies after the decrease of actin-mRNA levels, but before a significant amount of actin was cleaved (Guénal et al., 1997). Likewise, aggregation of actin without subsequent proteolytic cleavage has been observed in non-small cell lung cancer cells (van Engeland et al., 1997). Kruidering et al. (1998) observed loss of F-actin and an increase in soluble G-actin during cis-platin-induced apoptosis of renal tubular cells. Most recently, a possible causal link between depolymerization of F-actin and eventual cell death has been established by Korichneva and Hämmerling (1999): in murine EL4 T lymphoma cells, disorganization the actin cytoskeleton by cytochalasin B renders the cells more sensitive to anhydroretinol-induced cell death; conversely, stabilization of the actin filaments by jasplakinolide promotes cell resistance to apoptosis.

It should be noted that morphological changes during apoptosis such as DNA condensation and fragmentation as well as membrane blebbing have been associated with the activity of caspase-3 (for a review, see Porter and Jänicke, 1999). MCF-7 cells were found to lack functionally active caspase-3, and TNF-α- as well as staurosporine-induced death of MCF-7 cells was not associated with DNA fragmentation, cell shrinkage and blebbing (Jänicke et al., 1998). Therefore, 'classical' apoptosis might not ensue in MCF-7 cells upon antiestrogen treatment. However, autophagic death of glioblasoma cells induced by overexpression of Ras was revealed not to be due to the lack of caspases (Chi et al., 1999). Likewise, preliminary results indicate the formation of autophagic vacuoles preceding TAM-induced cell death in MCF-7 cell clones containing active caspase 3 (kindly provided by Drs R. U. Jänicke and A. G. Porter; W. Bursch, Ch. Gerner and U. Fröhwein, unpublished observation). Even for 'classical' apoptosis, alternative cell death pathways such as those involving caspase 7 or even without activation of any of the known caspases seem to exist (Berndt et al., 1998; for reviews, see Kitanaka and Kuchino, 1999; Porter and Jänicke, 1999). Therefore, the induction of autophagic death of MCF-7 cells by TAM is unlikely due to the lack of caspase 3. Rather, autophagic cell death seems to be a biological phenomenon of general importance in physiological and disease states (Anglade et al., 1997; Beaulaton and Lockshin, 1977; Cataldo et al., 1995; Clarke, 1990; Cornillon et al., 1994; D'Herde et al., 1996; Jochova et al., 1997; Kitanaka and Kuchino, 1999; Migheli et al., 1997; Schwartz et al., 1993; Schweichel and Merker, 1973; Zakeri et al., 1995).

In conclusion, the present study revealed profound differences in the fate of the cytoskeleton during apoptosis and autophagic cell death. During apoptosis in a number of biological systems, cytoskeletal proteins depolymerize or are already cleaved in early stages. In contrast, during autophagic cell death, as exemplified by tamoxifen-treated MCF-7 cells, the cytoskeleton is largely preserved even beyond the stage of nuclear destruction; presumably the cytoskeletal network is required for the initiation and progression of autophagocytosis serving to eliminate cytoplasmic constituents such as endoplasmatic reticulum, Golgi apparatus etc. during the cell's suicide. The present findings add support to existence of different pathways through which cells can execute programmed death; closer analysis of the differences may provide new targets for the control of PCD in various diseases.

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