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The Apoptotic Process of Human Bladder Carcinoma T24 Cells Induced by Retinoid

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Key Words
Apoptosis, T24 cells · Cytoskeleton · Mitochondria · Bax protein · T24 cells, apoptosis

Abstract
Breakdown of the cytoskeletal network and redistribution of cytoplasmic organelles are early events of programmed cell death. Previous studies showed that retinoic acid induces programmed cell death in many tumor cell lines and that cytokeratins, particularly cytokeratin 18, are affected in the early events of apoptosis. In this study, patterns of cytoplasmic intermediate filaments (cytokeratin 18), actin filaments, and microtubules, as well as Bax and Bcl-2 proteins in human bladder carcinoma T24 cells were examined before and after retinoic acid treatment by immunocytochemistry and conventional electron microscopy. Our results demonstrate that the redistribution of Bax and Bcl-2 proteins in the subcellular compartment of T24 cells is correlated with reorganization of the cytoplasmic intermediate filament network and that cytokeratins are cleaved by caspases, as revealed by the M30 antibody which recognizes a specific caspase cleavage site within cytokeratin 18. The cytoskeletal architectures of microtubules are not significantly affected in the early apoptotic process, from our observations. We suggest that the breakdown in the intermediate filament network associated with the aggregation of mitochondria and lysosome may be a crucial event in the apoptotic process and that aggregation of cytoplasmic Bax may accelerate apoptotic death.

Introduction

Apoptosis is a mode of cell death morphologically described in different sequentially developing steps, including membrane blebbing, perinuclear condensation of chromatin, nuclear fragmentation, and packaging of the nuclear fragments into membrane-enclosed apoptotic bodies [29]. Changes in the cytoskeletal organization were described as occurring during apoptosis [2, 20, 28]. It was demonstrated that cytokeratin intermediate filaments aggregate at an early stage of the apoptotic process, while at a later stage, cytokeratin is degraded [2, 17, 27]. It was suggested that morphological changes in the cytoskeleton, as demonstrated by a specific M30 antibody which recognizes a caspase cleavage site within cytokeratin 18, could be an early event of the apoptotic cascade [8, 11]. Recently, the degradation of cytokeratin 18 was further characterized not only at the M30 site but also at an additional internal site during apoptosis [9, 10].

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Retinoids are reported to exert their antitumor properties via apoptosis in neuroblastomas [18] and B cell lymphomas [25]. Mechanisms of apoptotic pathways have not been entirely elucidated and may involve a number of cellular proto-oncogenes, including *bcl-2* and *bax*, *bcl-x*, and other family members. Bcl-2 protein has been implicated as a suppressor of apoptosis in many cell types [7] and appears to function in the endoplasmic reticulum as well as in mitochondrial and nuclear membranes [3, 13, 26]. It was also reported that Bcl-2 selectively binds to other family members to regulate the induction of apoptosis [16, 30]. The ability of cells to undergo retinoid-induced apoptosis depends on the level of expression and the functional interaction between Bcl-2 and Bax [1]. The subcellular Bax localization changed from a diffuse pattern to a concentrated one in specific cytoplasmic areas at a stage preceding the formation of apoptotic bodies in an acute promyelocytic leukemia cell line [1].

In order to explore crucial events in the early apoptotic process, in the present study, we investigated cytoskeletal changes in cytokeratin intermediate filaments, actin filaments, and microtubules after retinoid-induced apoptosis, using the human bladder carcinoma T24 cell line as a model, by fluorescence immunocytochemistry and conventional electron microscopy. The subcellular localization of Bcl-2 and Bax before and after retinoid-induced apoptosis of T24 cells was also examined.

Materials and Methods

Cell Culture

The T24 cell line was obtained from ATCC (Rockville, Md., USA). Confluent cells were treated with all-trans-retinoic acid (RA; Sigma, St. Louis, Mo., USA) from 1×10^{-4} to 1×10^{-6} *M* for 24 h. After treatment, all surviving cells were collected for the experiments.

Antibodies

The following antibodies were commercially obtained and applied in the present study: (1) polyclonal antibodies to the oncoproteins, Bax and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA); (2) monoclonal antibodies to α-tubulin and cytokeratin 18 (Sigma), and (3) a special monoclonal antibody, M30, that recognizes the caspase-cleaved cytokeratin 18 which is used to detect the early apoptotic caspase activity (Roche, Mannheim, Germany).

Immunocytochemistry

T24 cells were fixed for 15 min in cold methanol at −20°C and rinsed three times in phosphate-buffered saline (PBS) prior to staining. All primary antibodies were applied for 2 h at room temperature and then rinsed five times for 3 min each with PBS. For the secondary antibodies, fluorescein-conjugated goat antirabbit IgG and rhodamine-conjugated rabbit antigoat IgG (Sigma) were applied at a 1:200 dilution in PBS for 1 h. After rinsing in PBS, the cells were mounted and examined with an Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Organelles and Nuclear Staining

Before and after RA treatments, T24 cells were stained with MitoTracker and/or LysoTracker according to protocols suggested by the manufacturer (Molecular Probes, Eugene, Oreg., USA). The cells were then fixed and processed for immunocytochemistry and nuclear staining with Hoechst 33342 (Sigma). Images of T24 cells with triple staining were acquired using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Western Blot Analysis

Cells were collected and resuspended in 1 ml SDS-PAGE buffer (0.1% SDS, 25 m*M* Tris, and 250 m*M* glycine). Cell lysates were then passed through a 26-gauge needle several times, boiled for 10 min, and centrifuged. Aliquots of the supernatant were run on a 7.5% PAGE gel and electrotransferred to nitrocellulose. Membranes were hybridized with Bax and Bcl-2 antibodies. Detection was performed using an alkaline phosphatase conjugated secondary antibody, an enzymatic chemiluminescence ECL kit (Amersham, Little Chalfont, UK), and autoradiography.

Electron Microscopy

For conventional electron microscopy, the cells were fixed with 5% glutaraldehyde in PBS for at least 2 h at 4°C and then rinsed three times in PBS prior to postfixation with 1% OsO_4 in 0.01 *M* PBS (pH 7.4), dehydrated in a graded series of ethanol (50, 70, 85, 95, and 100%), and embedded in an Epon-Araldite mixture. Ultrathin sections were obtained and stained with uranyl acetate and lead citrate before examining with a model 7100 electron microscope (Hitachi, Tokyo, Japan).

DNA Fragmentation

DNA was extracted from RA-treated and RA-untreated T24 cells by the phenol-chloroform method modified from the protocol suggested by Sambrook and Russell [23]. Gel electrophoresis was used to examine the DNA fragmentation.

Terminal Deoxynucleotidyl Transferase-Mediated Biotinylated Deoxyuridine Triphosphate Nick End Labeling (TUNEL)

RA-treated T24 cells were incubated with proteinase K (10 mg/ml; Roche) for 10 min at room temperature. End labeling was carried out with a digoxigenin-labeled TUNEL kit (Roche) according to the manufacturer's instructions. After incubation at 37°C for 15 min, the cells were rinsed in 2 × SSC for 15 min and blocked with 5% normal goat serum in Tris buffer (pH 7.4). The cells were then incubated with anti-digoxigenin-peroxidase antibody (1:100; Roche) at 4°C for 2 h and subsequently incubated with biotinylated secondary antibody made in appropriate species for 1 h and with the avidin-biotin complex for another hour. The reaction product was demonstrated by 3,3′-diaminobenzidine (Sigma). For the positive control, T24 cells were treated with DNase (0.5 μg/ml) prior to TUNEL staining.

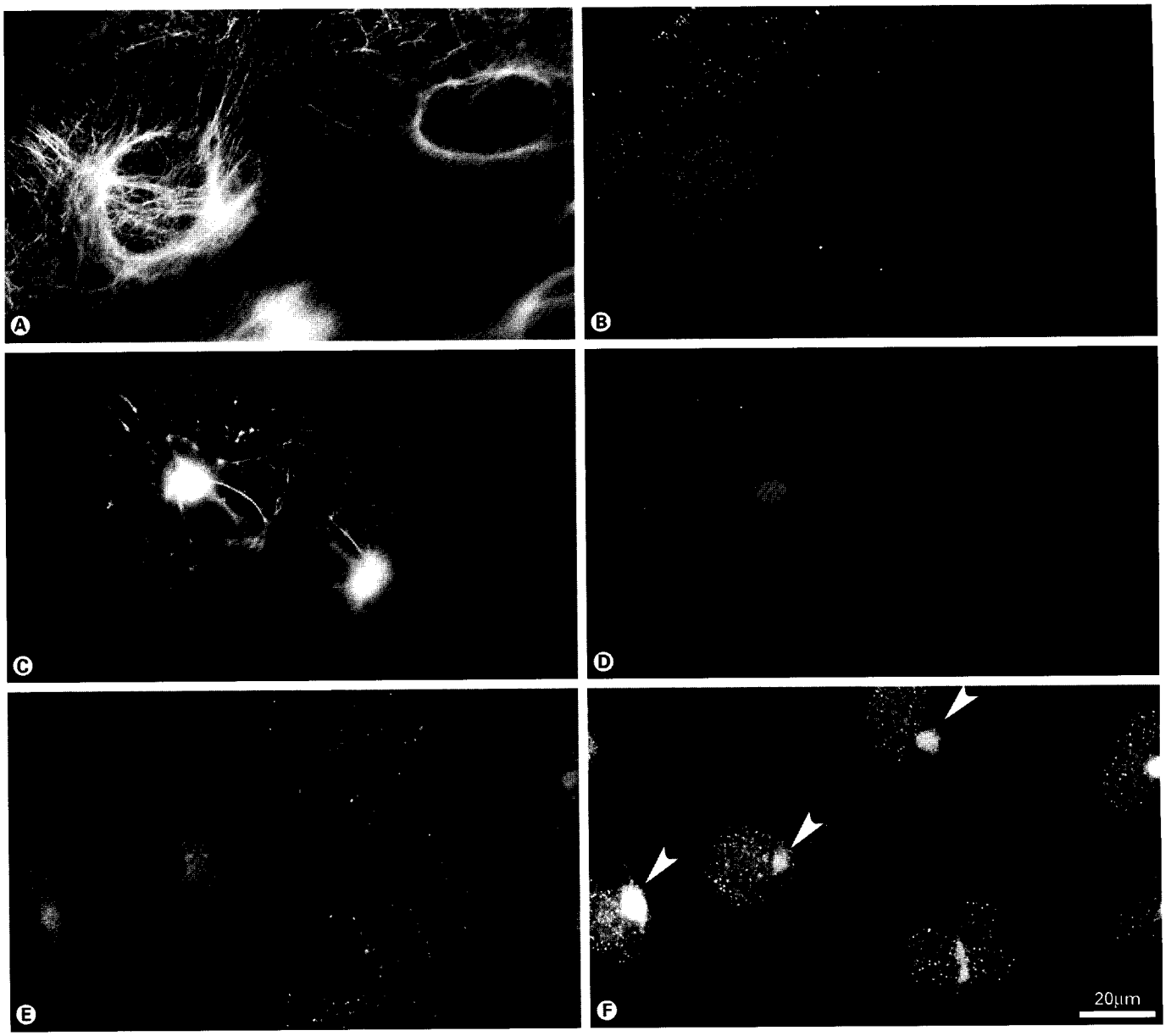


Fig. 1. Immunofluorescent localizations of cytokeratin 18 (K18), caspase-modified K18, and Bax protein in control (**A**, **B**) and in RA-treated T24 cells (**C–F**). The cells were stained with monclonal antibody to cytokeratin 18 (**A**, **C**), the M30 monclonal antibody that recognizes a specific caspase cleavage site within cytokeratin 18 (**E**), and with the polyclonal antibody for the Bax protein (**B**, **D**, **F**). Intermediate filament networks were broken (**C**), and enlarged cytokeratin 18 aggregates were very close to the Bax aggregates in RA-treated cells (**C**, **D**). Note the presence of Bax aggregates (arrowheads in **F**) and clear granular cytoplasmic staining with M30 antibody in RA-treated cells (**E**, **F**). Scale bar = 20 μm.

Results

Intermediate Filament Cytokeratin 18 Is the First Target in the Early Apoptotic Process

After 24 h of RA treatment, more than one third of the human bladder carcinoma T24 cells had died and were floating on the culture medium. The cells remaining attached to the cover glass slides were collected and fixed for immunocytochemistry.

The basic morphology of T24 cells attached to the cell culture dish still remained in good condition. In the double immunofluorescence staining of antibodies to cytokeratin 18 and Bax, the intermediate filament networks of RA-treated cells were seriously damaged as compared

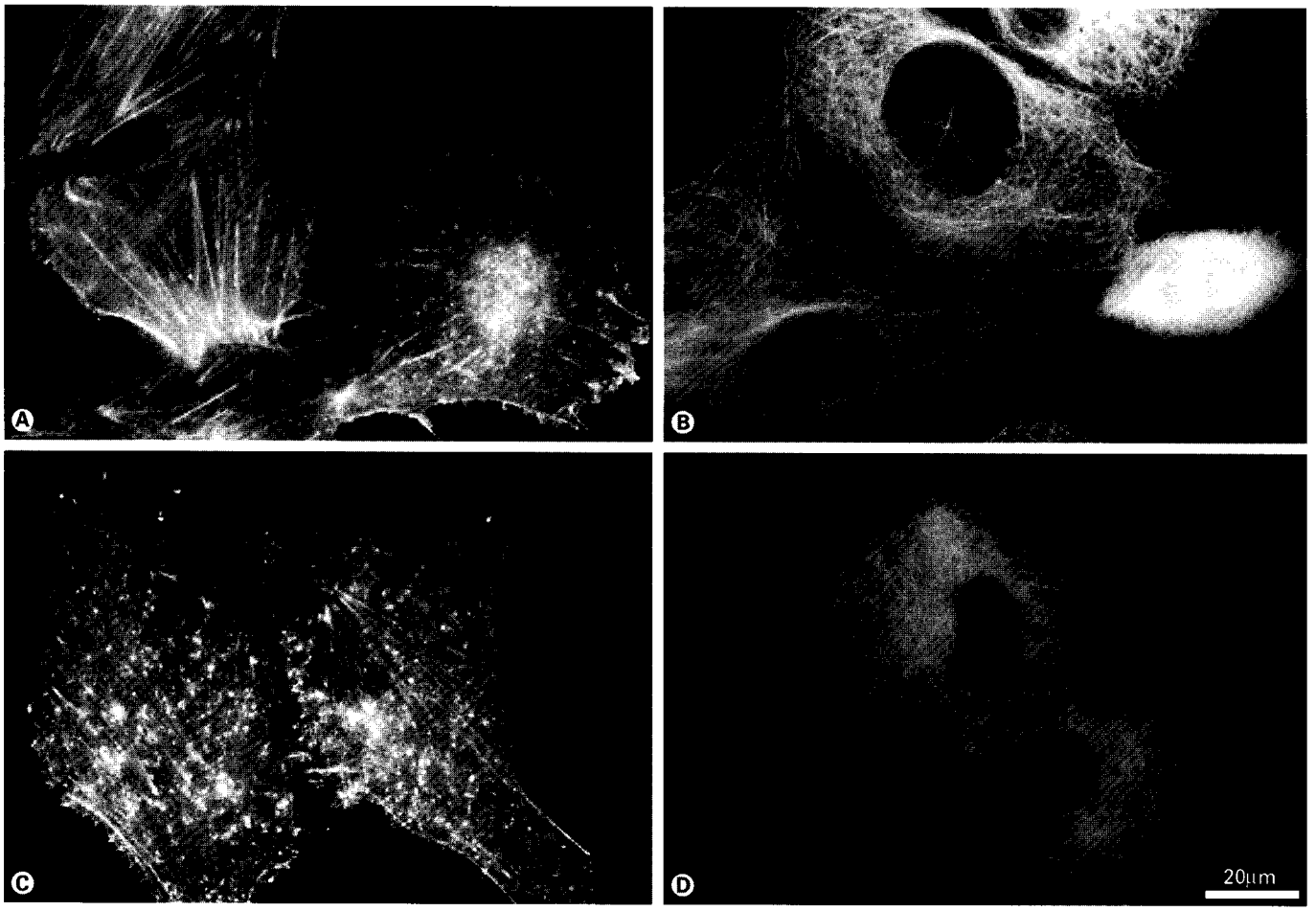


Fig. 2. Cytoplasmic patterns of actin filaments and microtubules in control (**A**, **B**) and RA-treated T24 cells (**C**, **D**). The cells were stained for actin filaments with rhodamine-conjugated phalloidin (**A**, **C**) and for microtubules with monoclonal antibody to α-tubulin (**B**, **D**). The actin stress fibers were less prominent with formation of a punctate pattern, as found in RA-treated cells (**C**). The cytoskeletal organization of microtubules remained intact after RA treatment (**D**). Scale bar = 20 μm.

with those of control T24 cells (fig. 1A, C). In RA-treated cells, intermediate filament networks were broken down, and the increasing sizes of the filamentous aggregations abutted the nucleus (fig. 1C). We also found that the distribution of the subcellular Bax protein had changed from a diffuse pattern to a concentrated locus in a specific cytoplasmic area near the cell nucleus (fig. 1B, D). It is worth mentioning that the Bax protein had amassed very close to the intermediate filament aggregation (fig. 1C, D).

To confirm that the disassembly of the intermediate filament network was a consequence of the caspase-activated apoptotic process, a special monoclonal antibody, M30, that recognizes caspase-cleaved cytokeratin 18, was also applied. In control T24 cells, no significant staining of antibody M30 could be detected. In the other case, both Bax aggregation and positive granular staining with the M30 antibody were observed in RA-induced apoptotic T24 cells (fig. 1E, F). However, the cytokeratin aggregates were devoid of M30 immunoreactivity (fig. 1E). The staining pattern of the M30 antibody in apoptotic T24 cells was almost the same as the pattern observed in MR65 squamous lung cancer cells [11].

Patterns of Actin Filaments and Microtubules in the T24 Subcellular Compartment

To examine patterns of actin microfilaments and microtubules in T24 cells before and after RA treatment, fluorescence staining of rhodamine-conjugated phalloidin

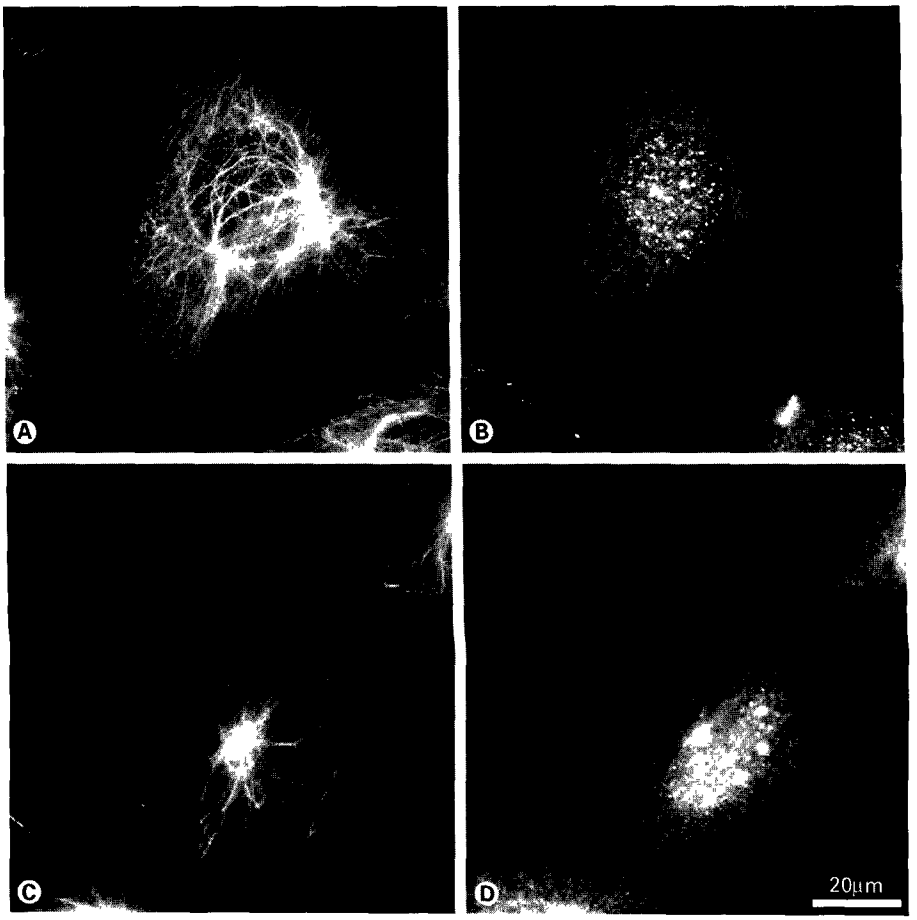


Fig. 3. Double immunofluorescent localization of cytokeratin 18 (K18) and Bcl-2 in control T24 cells (**A**, **B**) and RA-induced apoptotic T24 cells (**C**, **D**). Intermediate filaments of control and RA-treated cells were stained with the monclonal antibody to cytokeratin 18 (**A**, **C**) and with the polyclonal antibody for the Bcl-2 protein (**B**, **D**). An incomplete filamentous network and dense cytokeratin aggregates were demonstrated by the antibody to K18 (**C**). Subcellular localization of the Bcl-2 protein also changed from a diffuse pattern to concentrated aggregates (**B**, **D**). Scale bar = 20 μm.

and immunofluorescent staining of antibody to α-tubulin were applied. With the staining of rhodamine-conjugated phalloidin, the structure of actin microfilaments in T24 cells was affected after RA treatment; stress fibers were less prominent with formation of a punctate pattern (fig. 2A, C). Reorganization of the actin cytoskeleton was also observed in caspase-dependent apoptotic cells due to the cleavage of plectin, a cytoskeletal cross-linker protein [24]. On the other hand, immunofluorescent staining of α-tubulin revealed that the basic microtubule cytoarchitecture was not significantly affected after RA treatment (fig. 2B, D).

The Distribution of Cytoplasmic Bcl-2 Changed Together with the Breakdown of Cytokeratin

In the double immunofluorescent staining of antibodies to cytokeratin 18 and Bcl-2 (fig. 3), we observed that the subcellular Bcl-2 protein localization also changed from a diffuse pattern in cytoplasm and on the nuclear envelope to a few concentrated loci in some areas near the cell nucleus (fig. 3B, D). The cytokeratin network of T24 cells was also damaged after RA treatment (fig. 3A, C).

Aggregates of Bax and Bcl-2 Proteins Localized in Mitochondrial and Lysosomal Granules

T24 cells were vitally stained with MitoTracker green and LysoTracker red together with Hoechst stain before and after RA treatment (fig. 4). Distributions of mitochondria (fig. 4A, E) and lysosomes (fig. 4B, F) changed from being dispersed to aggregated patterns after RA treatment. These mitochondrial and lysosomal aggregates were localized on the concave surface of nuclei of RA-treated T24 cells (fig. 4G, H). This means that some mitochondria (green) were colocalized with lysosomes (red) (fig. 4D, H, overlaid with yellow). In further studies with immunostaining of Bax or Bcl-2 together with MitoTracker red and Hoechst stain, aggregates of Bax (fig. 5A) or Bcl-2 (fig. 5E) could be also found on the concave surface of nuclei (fig. 5G, H) and partially colocalized with mitochondria (fig. 5B, F) in RA-treated apoptotic T24 cells.

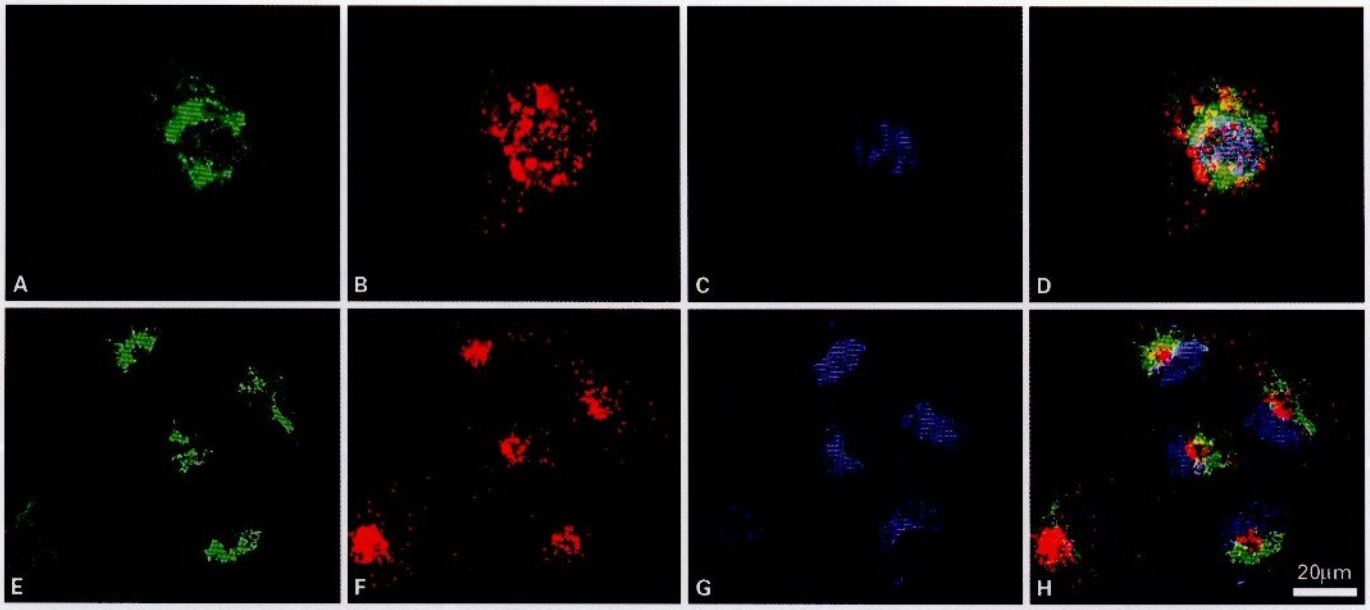


Fig. 4. Vital staining of T24 cells with MitoTracker, LysoTracker, and Hoechst 33342 before and after RA treatment. Mitochondria (**A**) and lysosomes (**B**) are widely distributed in healthy T24 cells with oval nuclei (**C**) before RA treatment. Both mitochondria (**E**) and lysosomes (**F**) aggregated on the concave surfaces of nuclei (**G**) of T24 cells after RA treatment. Note that some mitochondria colocalized with lysosomes in T24 cells before and after RA treatment (**D**, **H**, overlaid with yellow). Scale bar = 20 μm.

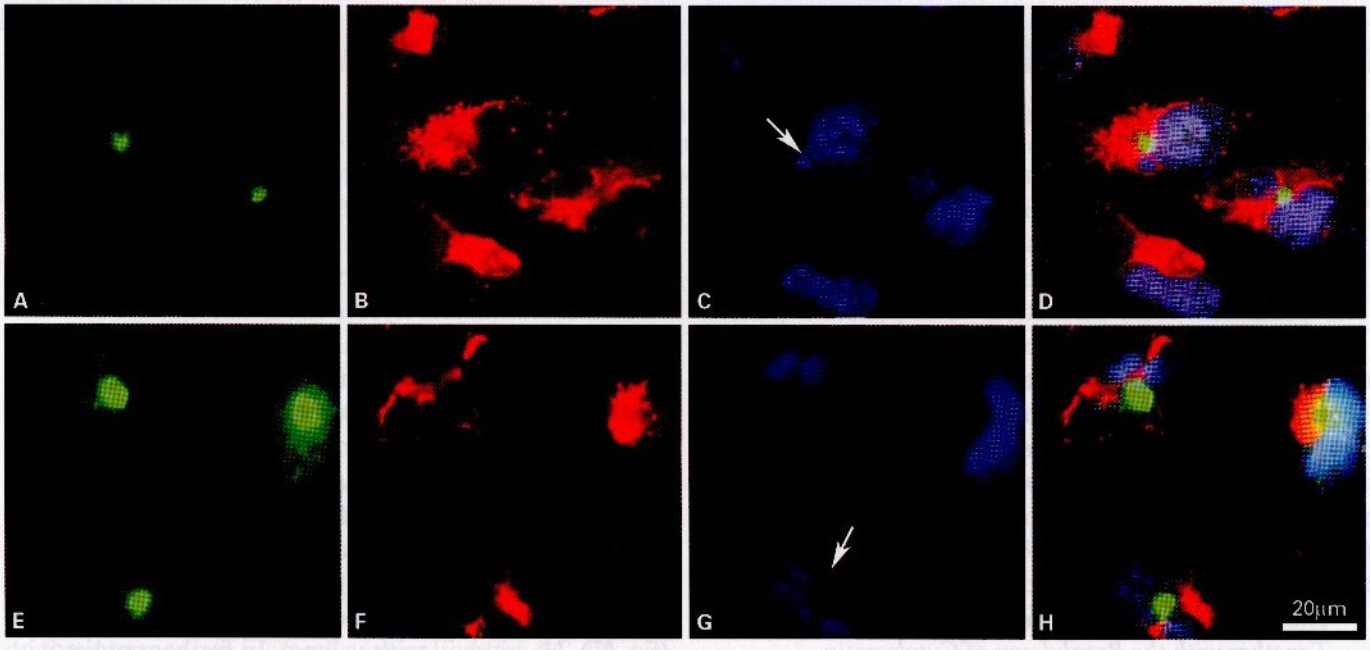


Fig. 5. Immunofluorescence of Bax and Bcl-2 together with MitoTracker red/Hoechst staining in RA-treated T24 cells. Both Bax (**A**) and Bcl-2 (**E**) in T24 cells were in an aggregate pattern after RA treatment. Not all of the Bax and Bcl-2 proteins colocalized with the mitochondria (**B**, **F**) in cells with apoptotic bodies (arrow in **C** and **G**). Scale bar = 20 μm.

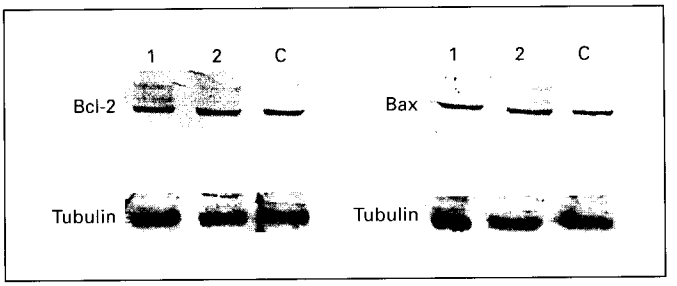


Fig. 6. Western blot analysis of Bcl-2 and Bax proteins from T24 cells pretreated with 1×10^{-6} *M* RA (lane 1) and 1×10^{-4} *M* RA (lane 2) for 24 h and the control (lane C). Blots were then probed with Bcl-2 and Bax antibodies and reprobed with the α-tubulin antibody as quantitative controls. There was no significant change in total protein levels of either Bcl-2 or Bax between the control and RA-induced apoptotic T24 cells.

The Amounts of Bcl-2 and Bax Proteins Not Significantly Changed by Apoptosis

In the Western blot assay, it was not possible to distinguish RA-treated T24 cells from untreated controls by the protein levels of Bcl-2 and Bax (fig. 6), even though the cytoplasmic Bax and Bcl-2 were relocated during the apoptotic process of RA-treated T24 cells.

Intermediate Filament Aggregation as well as Mitochondrial and Lysosomal Accumulation Distributed in the Indention of Nuclei of RA-Treated T24 Cells

Conventional electron microscopy revealed that control T24 cells are polyhedral in shape with an oval nucleus. Numerous mitochondria and lysosomes as well as bundles of intermediate filaments are randomly distributed in the cytoplasm (fig. 7A). An aggregated mass of intermediate filaments was localized near the indented nuclei in T24 cells after RA treatment (fig. 7B). Chromatin condensation and apoptotic body formation were also found in some RA-treated cells (fig. 7B). Moreover, mitochondria and lysosomes frequently accumulated in the deep nuclear indentation and near aggregations of intermediate filaments in RA-treated T24 cells (fig. 8A, B). Multilayer inclusions of lysosomes could be easily found around this mitochondria-rich region (fig. 8B). These ultrastructural observations confirm the data from immunofluorescent staining as well as organelle staining which implied that Bax- or Bcl-2-bound membranous organelles gathered very close to intermediate filament aggregations in T24 cells after RA-induced apoptosis.

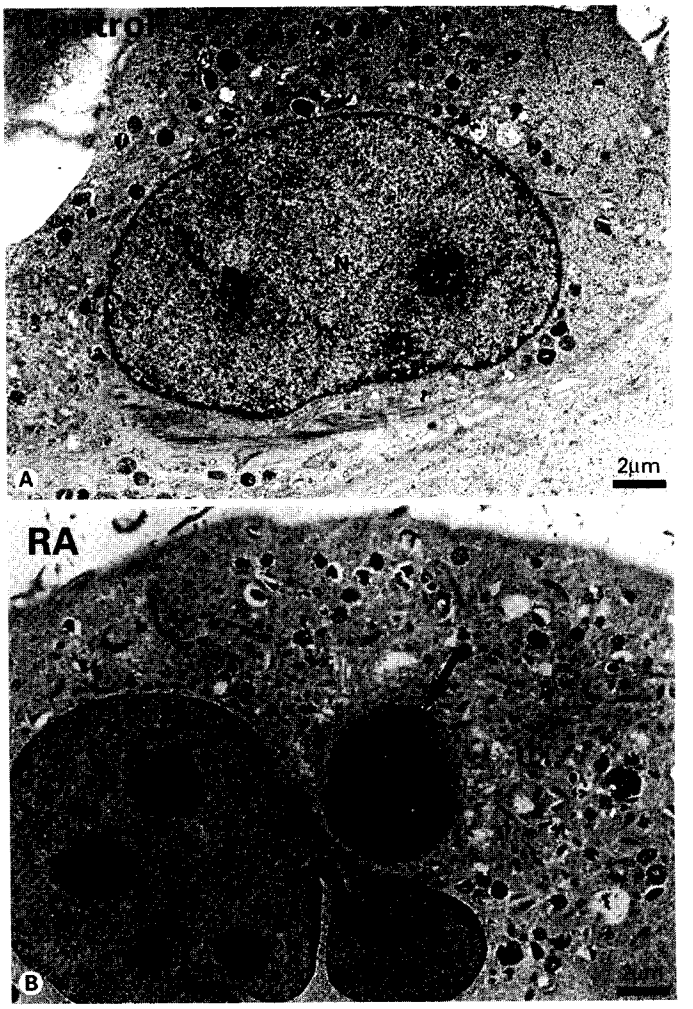
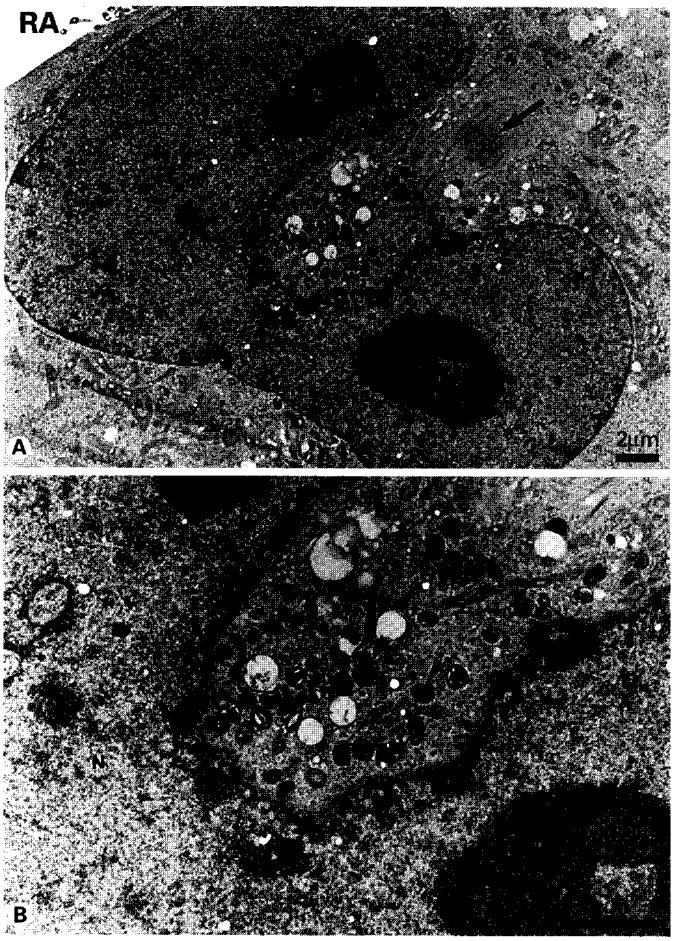


Fig. 7. Electron micrographs of control (**A**) and RA-induced apoptotic T24 cells (**B**). An oval nucleus, an abundance of mitochondria and lysosomes, as well as bundles of intermediate filaments can be observed in the cytoplasm of a control T24 cell (**A**). In the RA-treated cell, an enlarged intermediate filament aggregate (arrow) was localized close to the indented nucleus (**B**). N = Nucleus. Scale bar = 2 μm.

No Significant DNA Fragmentation in the Early Stage of Apoptosis Induced by RA

After electrophoresis, the running pattern of DNA extracted from RA-treated T24 cells was similar to that of DNA extracted from untreated T24 control cells. Nevertheless, DNA fragmentation could be detected in the collection of detached cells from the RA-treated T24 cell culture suspension.

After TUNEL staining, only a few RA-treated T24 cells were positively stained; most of the undetached cells were negative (fig. 9A). To monitor early signs of cell



apoptosis in RA-treated T24 cells, DNA fragmentation or positive TUNEL staining might not be as good as immunocytochemical detection of cytoskeletal breakdown.

Discussion

In the present study, we demonstrated that intermediate filaments as well as actin filaments are affected early in RA-induced apoptosis of T24 human bladder carcinoma cells. Numerous studies have shown that intermediate filament proteins, such as cytokeratin 18 and vimentin, are very sensitive to the apoptotic caspases [2, 15, 17, 19, 28]. It was demonstrated that although intermediate filament aggregation and proteolytic degradation as well as actin and tubulin reorganization occurred, the proteolytic cleavage of microfilaments and microtubules was not detected in the early apoptosis of human lung cancer and

Fig. 8. Electron micrographs of an RA-induced apoptotic T24 cell. An intermediate filament aggregation (arrow) is located very close to the nucleus (N), and numerous mitochondria and lysosomes are gathered in the indentation of the nucleus (**A**). At high magnification (**B**), many mitochondria, vacuoles, and lysosomes (arrowheads) were localized very close to the indented nucleus (B). Scale bar = 2 μm.

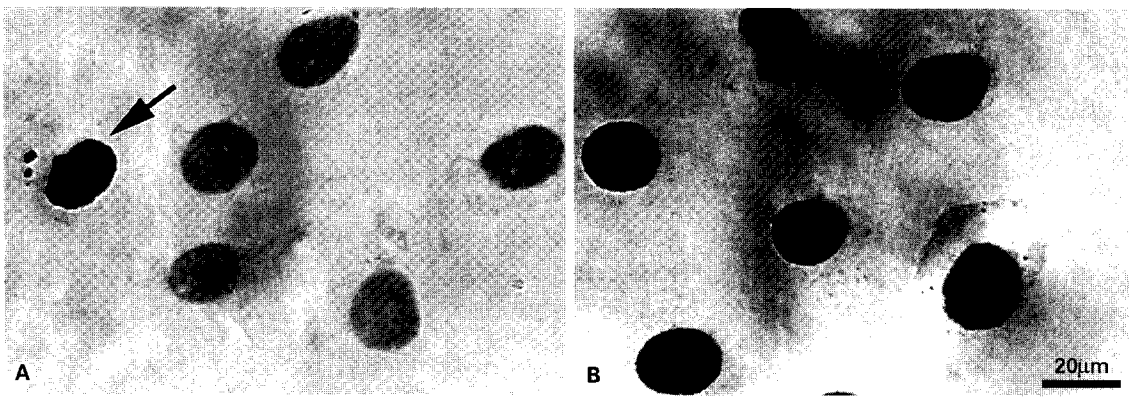


Fig. 9. TUNEL analysis of RA-treated T24 cells. Only a few apoptotic cells with apoptotic bodies (arrow) were strongly stained and could be observed by light microscopic survey (**A**). Positive TUNEL control was demonstrated by DNase-pretreated T24 cells (**B**). Scale bar = 20 μm.

neuroblastoma cells [28]. Nevertheless, proteolytic cleavage of plectin, a putative intermediate filament-actin microfilament cross-linker, may reorganize not only intermediate filaments but also actin microfilaments in the apoptotic cells [24]. In the present study of the early stage of RA-induced apoptosis in T24 cell lines, we observed that aggregation of cytoplasmic intermediate filaments and the pattern of actin microfilaments mildly changed, yet the organization of microtubules was not significantly altered. To detect apoptosis at an earlier phase, the application of antibody M30 is better than other methods such as the TUNEL assay or the annexin V assay [11]. Recent information also suggests that antibody M30 is superior to the TUNEL reaction as a marker for the early detection of apoptosis [5]. Our data obtained from the M30 antibody study are essentially similar to those of previous observations [11]. We provide evidence that cytoplasmic intermediate filaments may be one of the first substrates for the apoptotic caspase cascade in the early stage of programmed cell death.

A direct interaction between Bcl-2 and Bax in individual mitochondria has been demonstrated by two-fusion fluorescence resonance energy transfer [12]. In the present study, the cytoplasmic redistribution of Bax proteins was much more significant than that of Bcl-2 proteins, although the protein levels of neither Bax nor Bcl-2 significantly changed according to the Western blot assay. These data suggest that interactions of Bax/Bax and Bax/Bcl-2 at different cytoplasmic locations of mitochondria may be key for switching on the apoptotic cascade before the total protein levels of Bax or Bcl-2 change. This proposition is strongly supported by observations from another study [21] that Bax translocation is a critical event in neuronal apoptosis and that overexpression of Bcl-2 could not prevent Bax redistribution after apoptotic induction in neurons.

In this study, a dramatic change in the cytoplasmic localization of Bax proteins was observed after RA treatment. It was reported that Bcl-2/Bax family proteins exert their function on apoptosis only when they target the mitochondrial outer membrane [4, 12, 31]. Early in apoptosis, an important pathway leading to caspase activation involves the release of cytochrome *c* from the intermembranous space of mitochondria. Numerous investigations have shown that Bcl-2 and Bcl-2-related molecules exert their apoptotic regulatory efforts by facilitating or inhibiting the mitochondrial permeability transition pore opening, such as when Bcl-2 acts at the mitochondrial permeability transition pore to prevent Bax-mediated cytochrome *c* release [6, 14, 31]. Other data demonstrated that Bcl-2 indirectly protects mitochondrial membranes from Bax, via BH3-only proteins on the endoplasmic reticulum [26]. Bcl-2 may also regulate nuclear and cytosolic Ca^{2+} levels in its antiapoptotic functional role [13]. In this study, we found that aggregates of Bax and Bcl-2 remain a very short distance from the concave surfaces of nuclei. We speculate that apoptotic mechanisms may be triggered not only by the short-circuiting of Bax-mediated cytochrome *c* release from mitochondria, but also by the shuttling of Bcl-2 proteins between mitochondria and the nuclear envelope by way of the endoplasmic reticulum.

In the present study, both Bcl-2 and Bax aggregates were colocalized not only in mitochondria but also in lysosomes (revealed by confocal microscopy in figures 4 and 5), and some mitochondria were engulfed by lysosomes (multimembranous layers inside the lysosomes, as revealed by conventional electron microscopy in figure 8) after RA-induced apoptosis in T24 cells. From these observations, it can be hypothesized that these lysosomes may try to rescue the dying cell by swallowing up additional damaged Bax/Bcl-2-bound mitochondria. Nevertheless, it may be too late to stop the catastrophe due to limits of lysosomal activity. Technically to directly support this hypothesis, we should demonstrate that Bax/Bcl-2 aggregates colocalize with lysosomes. Yet the commercial LysoTracker is very sensitive to methanol or formaldehyde fixatives. The vanishing fluorescence of LysoTracker was not detected together with anti-Bax or anti-Bcl-2 immunofluorescence after fixation in our experimental test. In addition, antibodies against Bax or Bcl-2 might not recognize their specific epitopes after degradation within lysosomes.

So far, the mechanism responsible for mitochondria accumulation is an enigma, and intermediate filament aggregation occurred almost at the same subcellular location during RA-induced apoptosis of T24 cells according to our observations. It was reported [22] that plectin may also play a role as a cytolinker protein between intermediate filaments and mitochondria in striated muscle. During apoptosis, plectin is the major substrate for caspase 8 that predominantly colocalizes with and is bound to mitochondria, and the degradation of plectin precedes that of cytokeratin 18 [24]. Taking all these data together, we suggest that plectin plays an essential role in the T24 cell line during apoptosis. However, the role plectin plays between mitochondria and the cytoskeleton in apoptosis and apoptosis-independent cellular processes in T24 cells remains to be elucidated.

In summary, we conclude that in T24 human bladder carcinoma cells the intermediate filament cytokeratin is

one of the cytoskeletal components degraded by caspase after retinoid-induced apoptosis and that the redistribution of Bax and Bcl-2 in the subcellular compartment correlates with reorganization of the cytoplasmic intermediate filament network in the apoptotic process.

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