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Apoptosis of Ewing’s Sarcoma Cells Is Accompanied by Accumulation of Ubiquitinated Proteins

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Abstract

Induction of apoptosis in Ewing’s sarcoma cells by ionizing radiation is accompanied by accumulation of ubiquitinated proteins preferentially in the form of conjugates with Mr greater than 75,000. Furthermore, enhanced antiubiquitin immunofluorescence was detected only in cells that underwent radiation-induced apoptosis, suggesting that the observed alterations in protein ubiquitination are specific to the apoptotic process. To determine the role of the proteasome in apoptosis-associated accumulation of ubiquitin-protein conjugates, we used lactacystin, a highly selective inhibitor of proteasome proteolytic activity. Exposure of Ewing’s sarcoma cells to lactacystin resulted in accumulation of ubiquitinated proteins and activation of a Bcl-2-sensitive apoptotic pathways. The latter led to proteolytic cleavage of poly(ADP-ribose) polymerase and fragmentation of nuclear DNA. These findings suggest that proteasome function is required for apoptosis-specific accumulation of ubiquitinated proteins and indicate that functional disorder of the ubiquitin-proteasome system may play an important role in the apoptotic cell death pathway.

Introduction

The ubiquitin-proteasome system is a major nonlysosomal proteolytic pathway in eukaryotic cells (reviewed in Ref. 1). Ubiquitin, a small basic protein of 76 amino acid residues, is found in all eukaryotic cells. The coupling of ubiquitin to various target proteins is initiated by the ubiquitin-protein ligase system. Monoubiquitinated substrates then undergo additional modifications via the lysine residue at position 48 on the ubiquitin molecule, leading to the formation of multoubiquitin chains. The resulting ubiquitinated proteins are degraded by an ATP-dependent 26S proteasome complex; the proteolytic core of this structure contains multiple peptidase activities that function together during proteolysis (for reviews, see Refs. 1 and 2).

Recent studies have shown that ubiquitin is involved in the degradation, posttranslational modification, and activation of several important regulatory proteins. Oncoproteins and transcriptional regulators (p53, c-Fos, c-Jun, and NF-κB), cell surface receptors, cyclins, and cyclin-dependent kinase inhibitor p27Kip1 are included among these (1–4). Some proteins are degraded in a cell cycle-dependent manner, whereas others are subjected to limited proteolysis, resulting in posttranslational processing or degradation after ligand binding (1, 3, 4). Protein ubiquitination is not only essential for the normal physiological turnover of proteins but seems to have been adapted as part of an intracellular surveillance system that can be activated by altered, damaged, or foreign proteins (5–7). Several lines of evidence suggest the involvement of the ubiquitin system in mechanisms of neurodegenerative disorders and developmentally programmed cell death (Refs. 5 and 8 and references therein). Recent studies have demonstrated that the proteolytic activity of proteosomes is also essential for the induction of the apoptotic pathway, albeit with different outcomes for actively proliferating and quiescent or terminally differentiated cells (9–12). Moreover, functional and structural changes in the 26S proteasome complex have been found to occur specifically in cells undergoing programmed cell death (13, 14).

The present study addressed the question of whether the ubiquitin-proteasome pathway is involved in mechanisms of experimentally induced apoptosis in EWS3 cells. We report here that the induction of apoptosis in EWS cells by ionizing radiation is accompanied by an accumulation of ubiquitinated proteins. Furthermore, inhibition of proteasome function by lactacystin also results in the accumulation of protein-ubiquitin conjugates and triggers the Bcl-2-sensitive apoptotic pathway. Thus, functional alterations of the ubiquitin-proteasome system, resulting in accumulation of ubiquitinated proteins, seem to play an important role in apoptotic cell death.

Materials and Methods

Cell Culture and Transfections. The EWS cell line A4573 was kindly provided by Dr. Timothy Kinsella (University of Wisconsin Medical School, Madison, WI). Cells were maintained in Eagle’s MEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO2. A4573 cells were stably transfected with neomycin-selectable pRSor9-8-45 plasmid containing the human bcl-2 cDNA in sense orientation, or with control, neomycin-resistant expression vector pZIP-neo-SVX using LipofectAMINE (Life Technologies, Inc.) as described previously (15). The G418-resistant colonies from each replicade experiment were pooled and used in experiments as independent cell populations.

Cell Death Induction. Exponentially growing cells were irradiated at a dose of 7 Gy or exposed to 2.5–10 μM lactacystin for various intervals. Lactacystin (Calbiochem) was dissolved in DMSO and added to cell cultures at a final concentration of solvent in the medium not exceeding 0.1%. Control cells received DMSO only. All irradiations were performed at room temperature in air using a 137Cs source in a JL Shepard MARK I laboratory irradiator at a dose rate of 3.85 Gy/min.

Immunofluorescence Staining and Microscopy. For simultaneous staining of DNA and ubiquitin, EWS cells were grown on glass coverslips #1 (Fisher Scientific) placed in 35-mm wells of a 6-well culture dish. After treatment with drugs or irradiation, medium was removed, and cells were rinsed twice with PBS, fixed in PBS-3.7% paraformaldehyde for 10 min, washed three times in PBS for 5 min each, and then permeabilized with PBS-0.2% Triton X-100 for 10 min. After three washes with PBS, cells were incubated for 30 min with antiubiquitin antibodies (1:100; Chemicon). Washes were followed by incubation with affinity-purified Texas Red-conjugated donkey antirabbit IgG antibody (1:300; Jackson ImmunoResearch) and DAPI solution (0.5 μg/ml) for 30 min in the dark. Coverslips were then washed with PBS, blotted dry, and mounted onto glass slides using Prolong Antifade Kit

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3 The abbreviations used are: EWS, Ewing’s sarcoma; PARP, poly(ADP-ribbon) polymerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4,6-diamidino-2-phenylindole; ICE, interleukin-1β-converting enzyme; CED, Caenorhabditis elegans.
Analysis for Apoptosis. For quantitation of apoptosis, cells were washed with PBS before fixation with 3.7% paraformaldehyde for 3–5 min. After rehydration in PBS, cells were stained with DAPI (Sigma) for 30 min in the dark and visualized by fluorescence microscopy using a Zeiss Photoscope II. The apoptotic cells exhibiting condensed and fragmented nuclei were readily visible and scored positive. Using a ×40 objective lens, apoptotic nuclei were counted in five to seven randomly selected fields. A minimum of 400–600 nuclei were examined for each sample, and the results were expressed as the number of apoptotic nuclei over the total number of nuclei counted.

DNA Fragmentation Assays. For analysis of oligonucleosomal DNA fragmentation, total cellular DNA was prepared as described previously (16). Briefly, harvested cells were washed in PBS and resuspended in lysis buffer [10 mM Tris HCl (pH 8.0), 5 mM EDTA, 1% SDS, and 50 µg/ml RNase A] followed by incubation for 1 h at 37°C. Cell lysates were treated with proteinase K (100 µg/ml) in the presence of 1 M NaCl for 3 h at 37°C. DNA was extracted with phenol:chloroform:isoamyl alcohol, precipitated with ethanol, recovered after centrifugation, and resuspended in TE buffer [10 mM Tris HCl (pH 7.5) and 1 mM EDTA]. DNA preparations were end-labeled with Klenow fragments of DNA Polymerase I (US Biochemicals) and [32P]dCTP as described previously (16). Unincorporated nucleotides were removed by three consecutive precipitation cycles with ethanol. Recovered DNA samples were applied to 2% agarose gels and electrophoresed for 16 h at 1.5 V/cm. Gels were dried at 50°C and exposed to X-ray film. Flow cytometry was performed on cells fixed with ethanol, incubated in PBS for 20 min at 37°C, and resuspended in the DNA-staining solution containing 0.1% Triton X-100, 0.1 mM EDTA, 50 µM/ml RNase A, and 50 µg/ml propidium iodide in PBS. Cells were stored at 4°C until flow cytometry analysis using a FACS Star Plus flow cytometer (Becton Dickinson) as described previously (16). The cells with DNA content less than that of diploid cells in G0-G1 phase were considered to be apoptotic, and the percentages of these cells were calculated using Reproman software.

Western Immunoblot Analyses. Cells were washed twice with cold PBS and lysed at 4°C for 30 min in buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, and 20 µg/ml leupeptin). Insoluble material was removed by centrifugation at 4°C for 30 min at 16,000 × g, and protein concentrations were determined using the Micro BCA protein assay (Pierce). Cellular proteins (25–30 µg/sample) were resolved by SDS-gradient (10–20%) PAGE and immunoblotted with the monoclonal antiubiquitin antibody. The migration of appropriate molecular size markers (in kilodaltons) is indicated on the right. B, irradiated cells were stained with DAPI and visualized by fluorescence microscopy. Cells exhibiting condensed and fragmented nuclei were scored as apoptotic. The values are expressed in percentages and were determined as described in "Materials and Methods." Columns, mean; bars, SE determined from three independent experiments.

Fig. 1. Increased ubiquitin immunostaining is associated specifically with apoptotic cells. Apoptosis in A4573 EWS cells was induced by ionizing radiation (7 Gy). Chromatin condensation and ubiquitin immunostaining in A4573 EWS cells were examined 72 h after irradiation. Double staining of ubiquitin and DNA in A4573 cells was performed using antiubiquitin antibodies and DAPI as described in "Materials and Methods." The same fields were photographed at ×400 magnification under a Zeiss fluorescent microscope to visualize DAPI-stained nuclear DNA and immunofluorescence of ubiquitin. A representative experiment (of three) is shown.

Fig. 2. Radiation-induced accumulation of ubiquitin-protein conjugates and apoptosis in A4573 EWS cells. A, immunodetection of ubiquitinated proteins in EWS cells after radiation exposure (7 Gy). Cells were harvested at the indicated times after irradiation. Total cellular proteins (25 µg) were resolved by SDS-gradient (10–20%) PAGE and immunoblotted with the monoclonal antiubiquitin antibody. The migration of appropriate molecular size markers (in kilodaltons) is indicated on the right. B, irradiated cells were stained with DAPI and visualized by fluorescence microscopy. Cells exhibiting condensed and fragmented nuclei were scored as apoptotic. The values are expressed in percentages and were determined as described in "Materials and Methods." Columns, mean; bars, SE determined from three independent experiments.
Fig. 3. Lactacystin induces accumulation of ubiquitinated proteins and apoptosis in EWS cells. A, detection of ubiquitin-immunoreactive proteins (upper panel). Whole cell lysates were prepared from A4573 EWS cells treated with indicated concentrations of lactacystin for 24 h. Samples containing equal amounts of protein (25 μg) were resolved by SDS-gradient (10–20%) PAGE and immunoblotted with the monoclonal antiubiquitin antibody. The migration of appropriate molecular size markers (in kilodaltons) is indicated on the right. Immunodetection of the GAPDH is shown as a reference for protein loading equivalence (bottom panel). B, DNA content frequency histograms of control A4573 EWS cells (upper panel) and cells treated with 5 μM lactacystin for 24 h (bottom panel). The percentage of cells with hypodiploid DNA content is indicated. C, induction of oligonucleosomal-sized DNA fragmentation by lactacystin. A4573 EWS cells were treated with indicated concentrations of lactacystin for 24 h. Isolated DNA was analyzed by agarose gel electrophoresis. The migration of molecular size markers (in Kbp) is indicated on the right. D, PARP proteolysis in EWS cells treated with lactacystin for 24 h. Total cellular proteins (25 μg) were immunoblotted with the anti-PARP polyclonal antibody. The positions of native PARP (116 kDa) and the proteolytic cleavage product of PARP (85 kDa) are shown.

Results and Discussion

Accumulation of Ubiquitinated Proteins in Apoptotic EWS Cells. We have reported previously that EWS cells respond to ionizing radiation exposure by induction of apoptosis (15, 16). As shown in Fig. 1A, approximately 20–25% of EWS cells showed nuclei that were highly condensed and/or segmented within 72 h of treatment, consistent with the morphology of apoptosis (18). To determine whether the induction of apoptosis was accompanied by changes in protein ubiquitination, we examined the patterns of ubiquitin immunofluorescence. We found that cells that had a normal nuclear morphology showed a heterogeneous pattern of ubiquitin immunofluorescence. In contrast, cells that underwent radiation-induced apoptosis exhibited intense ubiquitin immunofluorescence (Fig. 1). No significant alterations in antiubiquitin fluorescence intensity were detected before the appearance of apoptosis-specific changes in nuclear morphology.

To further investigate the association of the ubiquitin pathway with the apoptotic process, we examined the extent of ubiquitin incorporation into protein conjugates in EWS cells after exposure to ionizing radiation. Immunoblot analysis of extracts from unirradiated (control) cells demonstrated the predictable pattern of ubiquitin-immunoreactive bands that formed a typical smear of slow-migrating ubiquitin conjugated proteins, as well as monomeric ubiquitin. After ionizing radiation, EWS cells exhibited accumulation of ubiquitinated proteins in the form of conjugates with Mr greater than 75,000 (Fig. 2A). The alterations in protein ubiquitination were observed approximately 48 h after irradiation and were coincident with an increased proportion of cells displaying apoptosis-specific nuclear morphology (Fig. 2B).

These data are consistent with the enhanced antiubiquitin immunofluorescence in apoptotic EWS cells (Fig. 1) and correspond temporally to radiation-induced internucleosomal DNA fragmentation and specific proteolysis of PARP (15, 16). Similar changes in the profiles
of ubiquitinated proteins were also detected in human breast carcinoma MCF-7 and MDA-MB-468 cell lines after induction of apoptotic cell death by etoposide, 5-fluoro-2'-deoxyuridine, and sodium butyrate.\(^4\) Taken together, these results suggest that alterations in protein ubiquitination are temporally associated with induction of the apoptotic program and occur specifically in apoptotic cells.

**Inhibition of Proteasome Function Results in Accumulation of Ubiquitin-Protein Conjugates and Apoptosis.** In living cells, ubiquitinated proteins are rapidly degraded by the 26S proteasome, a complex with multiple proteinase activity (for reviews, see Refs. 1 and 2). Therefore, abnormally high levels of ubiquitin-protein conjugates in dying cells may reflect the disturbed equilibrium between protein ubiquitination and proteasome-dependent degradation. We reasoned that failure of proteasome function can potentially contribute to accumulation of ubiquitin-protein conjugates in apoptotic cells. To determine the role of the proteasome in this process, EWS cells were exposed to lactacystin, a highly selective inhibitor of proteasome activity. Lactacystin is a Streptomyces metabolite that readily penetrates cell membranes and irreversibly blocks multiple proteasome activities (19). As shown in Fig. 3A, inhibition of proteasome function in EWS cells resulted in accumulation of ubiquitin-protein conjugates with Mr greater than 75,000 in a dose-dependent fashion. These observations are consistent with the interpretation that abnormal function of the proteasome may result in accumulation of ubiquitinylated proteins in apoptotic cells. Recently, structural and functional changes in 26S proteasome proteolytic complex have been found to occur specifically in muscles of Manduca sexta that are undergoing developmentally programmed cell death (13, 14). However, the mechanisms of these alteration(s) and its role in apoptotic cell death process remain to be elucidated.

We next investigated whether inhibition of proteasome function in EWS cells would result in induction of apoptosis. Flow cytometric analysis demonstrated that treatment of EWS cells with lactacystin resulted in an increase in the population of cells with fractional DNA content (Fig. 3B), consistent with the apoptotic process (16). Electrophoretic analysis revealed a pattern of internucleosomal DNA fragmentation, confirming that apoptosis was actually occurring in lactacystin-treated EWS cells (Fig. 3C). To determine whether inhibition of proteasome function is capable of triggering the apoptosis-specific activation of the ICE/CED-3 proteolytic pathway, we examined the extent of PARP cleavage in EWS cells. Consistent with previous reports on activation of PARP proteolysis in apoptotic EWS cells (15, 16), we observed the appearance of a characteristic 85-kDa fragment of PARP after induction of apoptosis by lactacystin (Fig. 3D). These findings suggest that lactacystin-induced apoptosis is associated with activation of CPP32 or other members of the ICE/CED-3 family of cysteine proteases (20).

Previously, we have demonstrated that overexpression of Bcl-2 suppresses radiation-induced proteolytic cleavage of PARP protein and apoptosis in EWS cells (15). To determine whether expression of Bcl-2 could modulate lactacystin-induced apoptosis, we stably transfected A4573 EWS cells with a full-length human bcl-2 cDNA (Fig. 4A). Heterologous, stable expression of Bcl-2 significantly inhibited lactacystin-induced apoptosis in a transfected pool of EWS/Bcl-2-1B cells as compared to similarly treated vector-transfected (Neo-1) EWS cells (Fig. 4B). To further investigate the involvement of Bcl-2 in regulation of lactacystin-induced apoptosis, we assessed the levels of proteolytic cleavage of PARP protein. Western blot analysis of cell extracts from EWS cells demonstrated that Bcl-2 strongly inhibited the cleavage of 116-kDa PARP protein into a characteristic 85-kDa fragment (Fig. 4C), suggesting that Bcl-2 functions upstream of activation of the ICE/CED-3 cysteine protease family in this cell death pathway.

In this study, we show that radiation-induced apoptosis in EWS cells is associated with accumulation of high molecular weight ubiquitin-protein conjugates and that failure of proteasome to degrade ubiquitin-protein conjugates also leads to accumulation of ubiquitinated proteins and activation of the apoptotic death pathway in EWS cells. These observations provide support for the hypothesis that functional disorder of the ubiquitin-proteasome pathway may play an important role in the regulation of apoptosis in mammalian cells.

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\(^4\) V. A. Soldatenkov, unpublished observations.
exact pathway(s) triggered by altered function of the ubiquitin-proteasome system remains to be investigated. Nevertheless, several interpretations for involvement of the ubiquitin-proteasome pathway in the apoptotic cascade can be considered. Proteasome activity is essential for cell cycle regulation (1, 4). Therefore, cell cycle arrest resulting from dysregulation of ubiquitin-proteasome function may apparently conflict with oncogene-driven stimuli to cell proliferation and subsequently direct tumor cells to the induction of apoptosis. This interpretation is supported by recent observations that inhibition of proteasome activity acts as a positive modulator of apoptosis in actively proliferating cells but does not trigger apoptotic cell death in quiescent, differentiated cells and in cells with low proliferative index (9–12). This clash hypothesis has also been implicated in radiation-induced apoptotic death of tumor cells (21). Alternatively, the deregulated function of the ubiquitin-proteasome proteolytic system may interfere with basal or stimuli-induced protein turnover, resulting in stabilization of cell death-promoting protein(s). For example, inhibition of the proteolytic degradation of $\kappa B$ results in induction of apoptosis in a B-cell lymphoma line (22). Moreover, enhanced ubiquitination and stabilization of the tumor suppressor protein p53 has been recently demonstrated in human osteosarcoma cells after radiation treatment (3). The turnover of both proteins, $\kappa B$ and p53, has been shown to be mediated by the ubiquitin-proteasome proteolytic pathway in living cells (3, 23).

In summary, our data demonstrate that radiation-induced apoptosis in EWS cells is associated with alterations in protein ubiquitination and suggest the involvement of the proteasome function in the accumulation of ubiquitinated proteins in apoptotic cells. Thus, functional disorder of the ubiquitin-proteasome proteolytic system may play an essential role in apoptotic cell death. Further identification and selective targeting of ubiquitin-dependent signaling pathways leading to apoptosis should facilitate our understanding of this process and its potential role in the treatment of cancer.

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References


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