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Sodium butyrate induces apoptosis and accumulation of ubiquitinated proteins in human breast carcinoma cells

Viatcheslav A. Soldatenkov[1,2], Sarada Prasad[1], Yaroslav Voloshin[1] and Anatoly Dritschilo[1]

[1] Department of Radiation Medicine, Division of Radiation Research, Georgetown University Medical Center, Washington DC 20007, USA
[2] corresponding author: Viatcheslav A. Soldatenkov, Department of Radiation Medicine, Georgetown University Medical Center, The Research Bld., Room E-207A, 3970 Reservoir Road, Washington, D.C. 20007-2197, USA
tel: (202) 687-1170; fax: (202) 687-2221;
e-mail: soldates@gunet.georgetown.edu

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Abstract

To investigate the possible relationship between apoptosis and the ubiquitin pathway we examined the patterns of ubiquitinated proteins in the human breast carcinoma MCF-7 cell line following induction of apoptotic death by sodium butyrate. Apoptosis in these cells was associated with internucleosomal DNA fragmentation and proteolytic cleavage of poly(ADP-ribose) polymerase. By dual *in situ* antiubiquitin immunofluorescence and chromatin DNA staining, we demonstrated that ubiquitin fluorescence was increased specifically in cells that underwent sodium butyrate-mediated apoptosis. The extent of ubiquitin incorporation into protein conjugates was examined in both adherent (not yet apoptotic) and floating (apoptotic) cell populations. We found that apoptotic cells exhibited enhanced intensity of ubiquitin-immunoreactive conjugates, whereas adherent cells did not. In addition, two-dimensional immunoblot analysis of proteins from apoptotic cells identified a set of isomeric ubiquitinated conjugates located at a pI range of 4.2–4.6 and a M_r approximately of 30 kDa. These data indicate that the ubiquitin pathway may play a role in the sodium butyrate-induced apoptotic program in breast carcinoma cells.

Keywords: apoptosis; sodium butyrate; ubiquitin; breast carcinoma

Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole; calcein-AM, calcein acetoxymethyl ester; PARP, poly (ADP-ribose) polymerase; ICE, interleukin-1β converting enzyme; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; 2D-PAGE, two-dimensional polycrylamide gel electrophoresis

Introduction

Several lines of evidence implicate the involvement of the ubiquitin system in selective degradation and posttranslational processing of functionally important proteins, including transcription factors, oncoproteins and cyclins (reviewed in: Goldberg, 1992; Ciechanover, 1994; Hochstrasser, 1996). In addition to the regulation of physiological protein turnover, ubiquitination appears to represent one of the major biochemical pathways involved in mechanisms of cellular pathology. The increase in the expression of the polyubiquitin genes and ubiquitin protein levels has been identified in human neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, diffuse Lewy body disease and amyotrophic lateral sclerosis (reviewed in: Mayer *et al*, 1991). Furthermore, recent studies indicate that ubiquitin plays a major role in the posttranslational modification and removal of proteins during physiological programmed cell death. For instance, the programmed neuromuscular cell death in hawkmoth, *Manduca sexta*, is accompanied by an increase of polyubiquitin gene expression (Schwartz *et al*, 1990). Ubiquitin levels were also increased at certain stages of development in dying muscles and neurons in *Drosophila*, neurons of *Manduca*, tunicate stomach, and interdigital cells of chicken embryos (reviewed in: Schwartz *et al*, 1993). These data suggest that a common molecular response occurs in dying cells which involves the ubiquitin system.

Cell-damaging agents, such as hyperthermia, ionizing radiation and chemotherapeutic drugs activate expression of polyubiquitin genes (Fornace *et al*, 1989; Delic *et al*, 1993), suggesting a role for the ubiquitin pathway in experimentally-induced apoptosis. Indeed, recent studies have demonstrated that the immunoreactivity of ubiquitin was increased in human peripheral blood lymphocytes (Delic *et al*, 1993) and Ewing's sarcoma cells (Soldatenkov and Dritschilo, 1997) undergoing radiation-induced apoptosis. Here we have investigated whether a potential relationship exists between protein ubiquitination and apoptotic cell death triggered by factors other than ionizing radiation, and whether apoptotic cells exhibit different profiles of ubiquitinated proteins. We report that sodium butyrate induces apoptosis in MCF-7 breast carcinoma cells, and that apoptotic cells exhibit altered patterns of protein ubiquitination. These results indicate that the ubiquitin pathway may play a role in experimentally-induced apoptosis.

Results

Sodium butyrate induces apoptosis in MCF-7 cells

Subconfluent cultures of logarithmically growing MCF-7 cells were treated with 3 mM sodium butyrate, a fatty acid salt that is known to inhibit cell proliferation and induce apoptosis in many tumor cell types at concentrations of 2–5 mM (Filippovich *et al*, 1988; Hague *et al*, 1993; Mandal and Kumar, 1996). We examined the kinetics of sodium butyrate-induced cell death by two-colour fluorescence cell viability

assay based on simultaneous visualisation of viable and dead cells with two fluorogenic probes, calcein-AM and ethidium homodimer-1. In viable cells nonfluorescent calcein-AM is converted by intracellular esterases to the intensively fluorescent (green) calcein. In contrast, ethidium homodimer is excluded by viable cells but enters cells that have lost membrane integrity. Upon binding to nucleic acids, the ethidium homodimer produces bright red fluorescence. Thus, viable cells exhibit green fluorescence, dead cells appear red, and interference of red and green colors results in yellow fluorescence in dying cells (Han *et al*, 1997). As shown in Figure 1, exposure of MCF-7 cells to sodium butyrate resulted in a pronounced and time-dependent decrease in cell viability. By 48 h of incubation, significant number of cells started rounding-up and were shed into the culture medium. The decrease in numbers of viable cells (green) was coupled with the appearance of dead cells (red) and accumulation of detached (floating) cells in medium. Most of the floating cells (about 80%) were permeable to ethidium homodimer and stained red, indicating ongoing cell death. In contrast, approximately 95% of the cells remaining attached were still viable as demonstrated by fluorescence activation of calcein-AM.

To test whether MCF-7 cells undergo apoptosis following exposure to sodium butyrate, DNA fragmentation analysis was carried out in both adherent and floating cells (Figure 2A). DNA isolated from floating cells demonstrated a ladder-like pattern characteristic of inter-nucleosomal fragmentation, a biochemical marker of apoptosis (Wyllie *et al*, 1980), whereas adherent cells retained high molecular weight DNA. Recently, proteases of the ICE/CED-3 family have been implicated in apoptosis induced by a variety of stimuli (reviewed in: Kumar, 1995). To determine whether sodium butyrate is capable of triggering the apoptosis-specific activation of the ICE/CED-3 proteolytic pathway, we examined the extent of PARP cleavage in MCF-7 cells. Consistent with the results of cell viability assays and DNA fragmentation analyses we observed the cleavage of 116 kDa PARP protein to yield a characteristic 85 kDa fragment of PARP in a population of floating cells (Figure 2B). Control (untreated) MCF-7 cells and cells remaining attached following exposure to sodium butyrate did not show evidence of apoptosis-specific proteolysis of PARP. These findings are in agreement with the reported capability of sodium butyrate to activate apoptotic pathways in mammalian cells (Filippovich *et al*, 1988; Hague *et al*, 1993; Mandal and Kumar, 1996), and provide the first evidence of the role for the ICE/CED-3 family of cysteine proteases in sodium butyrate-induced apoptosis of breast carcinoma cells.

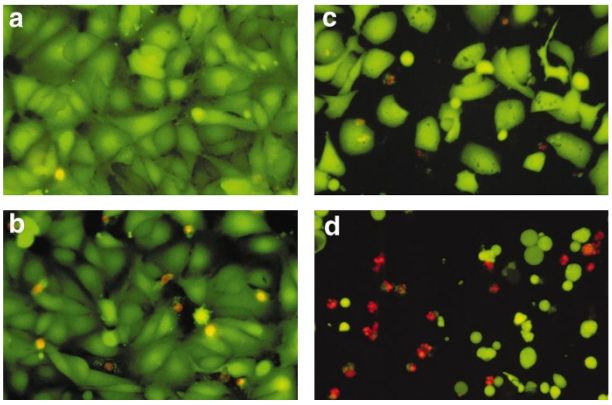

Figure 1 Effect of sodium butyrate on MCF-7 human breast carcinoma cells: induction of cell death. Cells were treated with 3 mM sodium butyrate for 0 h (**a**), 24 h (**b**) and 48 h (**c, d**). Floating cells (**d**) and cells remaining attached to substrate (**a, b, c**) were processed independently and stained with 2 μM calcein-AM and 4 μM ethidium homodimer-1 as described in Materials and Methods. Viable cells (green), dying cells (yellow), and dead cells (red) were detected by two-colour fluorescence cell viability assay and photographed under a Zeiss fluorescence microscope with exitation at 495 nm. Representative fields are shown

Sodium butyrate-induced apoptosis is accompanied by alterations in protein ubiquitination

To investigate whether induction of apoptosis was accompanied by changes in protein ubiquitination we examined patterns of ubiquitin immunofluorescence and nuclear morphology in MCF-7 cells. As shown in Figure 3, a heterogeneous pattern of ubiquitin immunofluorescence was

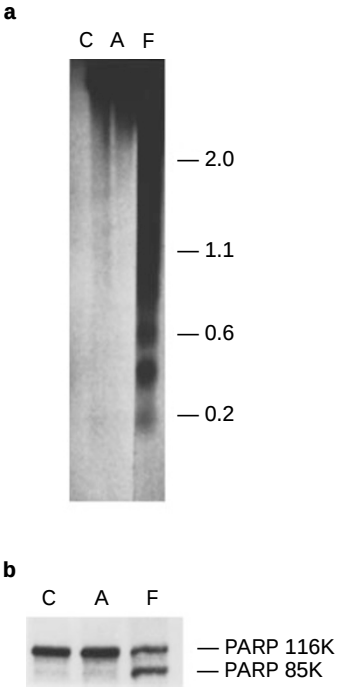

Figure 2 Sodium butyrate induces apoptosis in MCF-7 cells. Cells were treated with 3 mM sodium butyrate for 48 h. Floating (F) and adherent (A) cells were collected separately for subsequent analyses. Control, untreated cultures (C) contained less than 3% of floating cells and were analyzed in bulk. (**A**) Agarose gel electrophoresis of DNA. A typical 'ladder' pattern indicates internucleosomal DNA fragmentation in floating cells. The migration of molecular size markers (in kilobase pairs) is indicated on the right. (**B**) Immunodetection of PARP. Whole cell lysates were prepared from MCF-7 cells treated as indicated above. Cell samples containing equal amounts of protein (25 μg) were electrophoresed and subjected to immunoblotting with the anti-PARP polyclonal antibody. The positions of native PARP (116 kDa) and the proteolytic cleavage product of PARP (85 kDa) are shown

attributed to cells which had a normal nuclear morphology. In contrast, cells which revealed condensed nuclei, consistent with the morphology of apoptosis (Wyllie *et al*, 1980), also exhibited intense ubiquitin immunofluorescence. No significant alterations in antiubiquitin fluorescence intensity were detected prior to appearance of apoptosis-specific changes in nuclear morphology. These data indicate that apoptotic MCF-7 cells accumulate ubiquitin and/or ubiquitinated proteins. Increased levels of ubiquitin-protein conjugates were also observed in peripheral blood lymphocytes (Delic *et al*, 1993) and Ewing's sarcoma cells (Soldatenkov and Dritschilo, 1997) undergoing radiation-induced apoptosis, in lethally heated HeLa cells (Carlson *et al*, 1987) and during developmentally programmed cell death (reviewed in: Schwartz *et al*, 1993). Thus, accumulation of ubiquitin appears to be a common feature of cells dying by apoptosis.

Immunoblot analysis of ubiquitin conjugates in apoptotic MCF-7 cells

We next examined the extent of ubiquitin incorporation into protein conjugates in sodium butyrate treated cells. In consideration of the results reported above (Figures 1 and 2) separate analyses were carried out in adherent and detached cells. Ubiquitin is known to be present in free form (M_r 8000) and in association with various cellular proteins producing the conjugates with significantly higher molecular weights (Bush, 1984). Immunoblot analysis of MCF-7 cell extracts demonstrated the typical pattern of ubiquitin-immunoreactive bands that formed slow migrating ubiquitin-conjugated proteins. Following treatment with sodium butyrate, cells remaining attached did not show significant changes in the patterns of ubiquitinated proteins compared to control, untreated cells. In contrast, floating cells exhibited overall enhanced intensity of ubiquitin-immunoreactive bands, suggesting increased protein ubiquitination in apoptotic cells (Figure 4). Moreover, several ubiquitin-protein conjugates at M_r approximately of 30 kDa were accumulated specifically in floating cells. These data are consistent with the analyses of ubiquitin immunofluorescence in cells displaying an apoptosis-specific nuclear morphology (Figure 3), further strengthening the interpretation that alterations in protein ubiquitination occur specifically in apoptotic cells.

To further resolve the complex of ubiquitinated protein substrates, we employed two-dimensional gel electrophoresis following by immunoblot identification of ubiquitin-protein conjugates in attached and floating MCF-7 cells (Figure 5). The immunoreactive proteins are all concentrated in two groups, both in the acidic region at pI 4.2–4.6. Group I proteins resolve at an M_r range of 70–90 kDa while members of group II are located at an approximate M_r of 30 kDa. It is of interest that the group I protein acceptors of ubiquitin are present in both attached and floating cells, while the ubiquitin-protein conjugates of group II are detectable only in floating cells. The appearance of the additional set of 3–4 isomeric ubiquinated proteins in

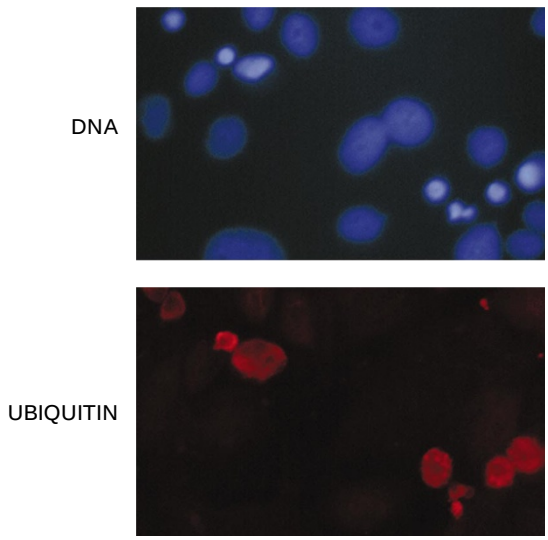

Figure 3 Increased ubiquitin immunostaining is associated specifically with apoptotic cells. Nuclear chromatin condensation and ubiquitin immunostaining in MCF-7 cells were examined following 48 h of treatment with sodium butyrate (3 mM). Double staining of ubiquitin and DNA in MCF-7 cells were performed using anti-ubiquitin antibodies and DAPI as described in Material and Methods. The same fields were photographed at $\times$400 magnification under a Zeiss fluorescent microscope to visualize DAPI-stained nuclear DNA and immunofluorescence of ubiquitin. A representative experiment (of three) is shown

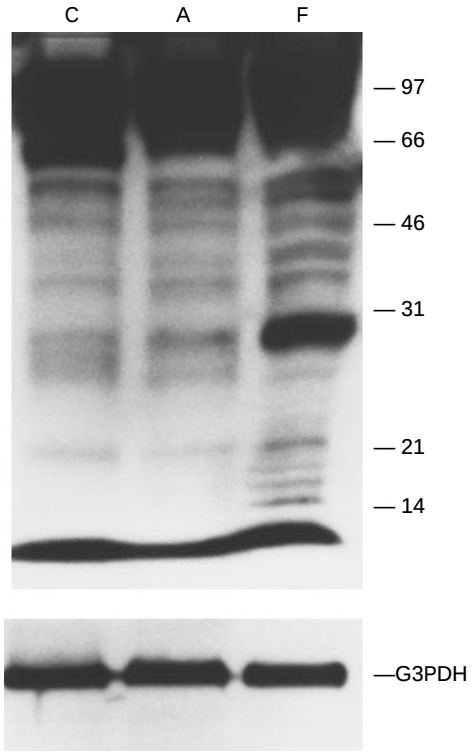

Figure 4 Detection of ubiquitin-immunoreactive proteins in MCF-7 cells exposed to sodium butyrate (3 mM) for 48 h. Floating (F) and adherent (A) cells were collected separately for analysis, and control cells (C) were analyzed in bulk. Total cellular proteins (25 μg) were resolved by SDS–PAGE (4–20% gradient gel), transferred to Immobilon-P membranes, and immunoblotted with the monoclonal anti-ubiquitin antibody (top panel). The migration of appropriate molecular size markers (in kilodaltons) is indicated on the right. Immunodetection of the G3PDH is shown as a reference for protein loading equivalence (bottom panel)

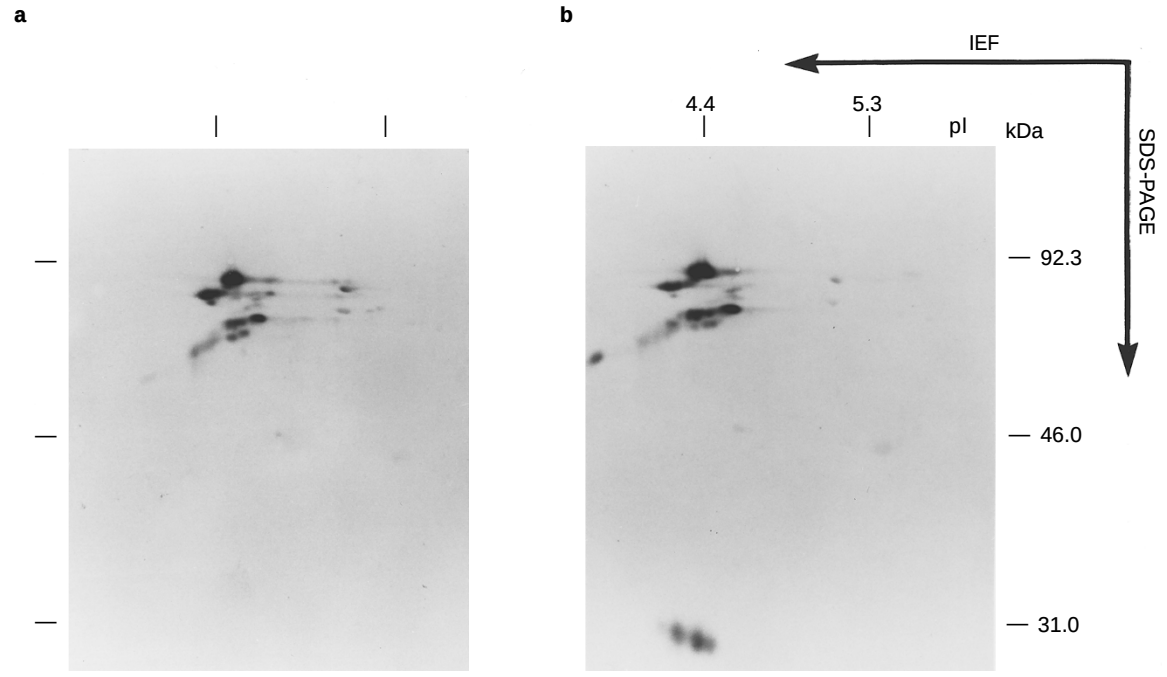

Figure 5 Two-dimensional immunoblot analysis of ubiquitin-conjugated proteins in MCF-7 cells. Following treatment with 3 mM sodium butyrate for 48 h adherent (**A**) and floating (**B**) cells were collected separately for two-dimensional electrophoretic analysis. Immunoblot identification of ubiquitinated proteins was performed using the monoclonal anti-ubiquitin antibody (Chemicon). The immunoreactive proteins are all concentrated in two groups, both in the acidic region at a pI 4.2–4.6. Group I protein acceptors resolve at an M_r range of 70–90 kDa while group II proteins are located at an M_r approximately of 30 kDa. The protein loading was equivalent in the gels used for immunoblotting as judged by comparable coomassie stain prior to using the membranes for subsequent immunodetection of ubiquitin

floating cell population indicates the specificity of these conjugates to apoptotic cells. At present it is unknown whether these protein isoforms represent intermediates in the ubiquitin-mediated proteolysis of protein targets or correspond to novel ubiquitinated products in the apoptotic pathway. Ubiquitin is a basic protein, and ubiquitination renders the protein acceptors to be more basic (Bush, 1984). Therefore, the acidic nature of these proteins suggests that in addition to ubiquitination they might have also undergone additional posttranslational modifications such as phosphorylation or glycosylation, or both. Recently the mechanistic link between site-specific phosphorylation of proteins and ubiquitination has been demonstrated (Chen *et al*, 1995). The identity of ubiquitin-immunoreactive proteins in apoptotic MCF-7 cells and any precursor product relationship between them are currently under investigation.

Discussion

We report here that sodium butyrate-induced apoptosis is associated with increased ubiquitin immunoreactivity in MCF-7 cells. Accumulation of ubiquitin-protein conjugates was observed specifically in cells which exhibited apoptotic morphology and biochemical criteria of apoptosis pointing to a possible relationship between function(s) of the ubiquitin pathway and the apoptotic process. In living cells, ubiquitinated proteins are rapidly degraded by the 26 S proteasome, a complex with multiple proteinase activity (reviewed in: Goldberg, 1992; Ciechanover, 1994). Therefore, accumulation of ubiquitin-protein conjugates in dying cells may reflect the disturbed equilibrium between protein ubiquitination and proteasome-dependent degradation.

Although the mechanisms leading to the observed alterations remain to be investigated, several explanations can be considered. First, protein ubiquitination appears to have been adapted as part of an intracellular surveillance system that can be activated by altered or foreign proteins (Carlson *et al*, 1987; Fornace *et al*, 1989; Mayer *et al*, 1991). Accordingly, the accumulation of ubiquitinated proteins may reflect the activation of the ubiquitin pathway as a part of the cytoprotective mechanism in chronically stressed and/or damaged cells. Second, abnormal function of the proteasome may also result in accumulation of ubiquitinated proteins in apoptotic cells. This interpretation is supported by our findings that inhibition of proteasome function in human tumor cells resulted in accumulation of ubiquitin-protein conjugates in a dose dependent fashion and activated apoptotic pathways in Ewing's sarcoma cells (Soldatenkov and Dritschilo, 1997). The role of the proteasome in the apoptotic process has also been demonstrated in other *in vitro* studies (Imajoh-Ohmi *et al*, 1995; Grimm *et al*, 1996; Saoul *et al*, 1996; Drexler, 1997). Moreover, structural and functional changes in the 26S proteasome occur during developmentally programmed cell death in muscles of *Manduca sexta* (Jones *et al*, 1995; Low *et al*, 1997). Third, the selective accumulation of ubiquitin-protein conjugates may be accounted for by increased

resistance of ubiquitinated proteins to proteolysis. Escape of ubiquitinated proteins from degradation is not unique since stable conjugates are found with histones (Wu *et al*, 1981), actin (Ball *et al*, 1987) and cell surface proteins (Siegelman *et al*, 1986). Moreover, accumulation of high molecular weight ubiquitin-protein conjugates was found to be coupled with acquired stabilization of ubiquitinated endogenenous and injected proteins in heat-shocked HeLa cells (Carlson *et al*, 1987).

Recent studies have demonstrated that the ubiquitin pathway is involved in the regulation of basal or stimuli-induced turnover of oncoproteins and transcriptional regulators (p53, c-*fos*, c-*jun*, and NF-κB), cell surface receptors, cyclins (reviewed in: Ciechanover, 1994) and the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Pagano *et al*, 1995). Accordingly, ubiquitin-mediated changes in the stability of functionally important proteins may play an essential role in apoptotic pathways. Further investigations are necessary to understand the mechanisms underlying stabilization of ubiquitinated proteins, to identify the apoptosis-specific protein targets for ubiquitin and resolve the questions of ubiquitinated protein targets in apoptosis. The latter may lead to the identification of critical intracellular mediators of apoptosis.

Materials and Methods

Cell culture and apoptosis induction

Human breast carcinoma MCF-7 cells were cultured in IMEM medium (Biofluids, Inc.) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained as monolayers at 37°C in an atmosphere of 5% CO_2. Exponentially growing cells were treated with sodium butyrate (Calbiochem) dissolved in medium to the indicated final concentration.

Two-color fluorescence cell viability assay

Cells were grown on sterile 18-mm glass coverslips (Fisher) placed in 35-mm wells of a six-well culture dish (Costar). Following treatment with 3 mM sodium butyrate, medium was removed and detached (floating) cells were collected by centrifugation. Floating cells and cells remaining attached to substrate were processed simultaneously according to manufacturer's protocol. Briefly, cells were washed with phosphate-buffered saline (PBS) and stained with 2 μM calcein-AM and 4 μM ethidium homodimer (LIVE/DEAD Viability/Cytotoxicity Assay Kit; Molecular Probes, Inc.) for 35 min. Labelled cells were immediately viewed and photographed under a Zeiss (Axiovert 135, Carl Zeiss Inc., Germany) fluorescence microscope with exitation at 495 nm.

Immunofluorescence staining and microscopy

For simultaneous staining of DNA and ubiquitin, MCF-7 cells were grown on glass coverslips placed in 35-mm wells of a six-well culture dish. Following treatment with 3 mM sodium butyrate, floating cells were spun down on coverslips and medium was carefully removed. 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 were permeabilized with PBS-0.2% Triton X-100 for 10 min. After three washes with PBS cells were incubated for 30 min with anti-ubiquitin antibodies (1 : 100, Chemicon). Washes were followed by incubation with affinity purified Texas Red-conjugated donkey anti-rabbit IgG antibody (1 : 300, Jackson ImmunoResearch) and DAPI solution (0.5 μg/ml, Sigma) for 30 min in the dark. Coverslips were then washed with PBS, blotted dry and mounted onto glass slides using a Prolong Antifade Kit (Molecular Probes, Inc.). Epifluorescence microscopy was performed using a Zeiss Photoscope II (Carl Zeiss, Inc.) equipped with a Zeiss Plan 40 $\times$/0.65 NA objective, and cells were photographed using a 35 mm camera. Specificity of ubiquitin immunofluorescence was confirmed by the absence of Texas-Red cell staining when the primary antibody was omitted. The apoptotic cells were underrepresented in these experiments due to the loss of floating cells following washes.

Analysis for apoptosis

For quantitation of apoptosis, adherent and floating cells were pooled together and washed with PBS prior to fixation with 3.7% paraformaldehyde for 3–5 min. After rehydration in PBS, cells were stained with DAPI for 30 min in the dark, mounted with coverslips 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 $\times$40 objective lens, apoptotic nuclei were counted in five to seven randomly selected fields. A minimum of 400–500 nuclei was examined for each case, 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 (Soldatenkov *et al*, 1995). Briefly, harvested cells were washed in PBS and resuspended in lysis buffer (10 mM Tris HCl, pH 8.0–5 mM EDTA–1% SDS–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 by centrifugation and resuspended in TE - buffer (10 mM Tris HCl, pH 7.5–1 mM EDTA). Recovered DNA samples were applied to 2% agarose gels and electrophoresed for 16 h at 1.5 V/cm.

2D–PAGE

Iso-Dalt equipment from Hoefer Scientific was utilized. Procedures were carried out essentially as described by O'Farrel (1975) with minor modifications to suit Iso-Dalt equipment. Isoelectric focussing gel tubes were made up of 3–5% acrylamide, 9 M urea, 2% ampholines (1 : 3 of pH 3.5–10.0 and pH 4.0–8.0), 2% nonidet-40 (NP-40), 0.03% ammonium persulfate, and 0.01% TEMED (N,N,N′N′-tetramethylene diamine). Total cell lysates were prepared in iso-sample buffer (5×10^6 cells in 250 μl) made up of 9 M urea, 4% NP-40, 2% ampholines (pH 8–10), and 1% dithiothreitol. 15–20 μl of whole cell lysates were subjected to 2D–PAGE. Proteins were focussed for approximately 24 000 V h (first dimension) and the isotubes transferred to 10% polyacrylamide slab gels for electrophoresis (second dimension) overnight at 100 V at 20°C. The gels were transblotted for subsequent immunoblotting in a semi-dry blotter (Hoefer Scientific Instruments) with a buffer of 25 mM Tris - 192 mM Glycine-20% methanol, pH 8.3.

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, 20 μg/ml leupeptin. Insoluble material was removed by centrifugation at 4°C for 30 min at 16 000 $\times$ *g* and protein concentration was determined using the Micro BCA protein assay (Pierce). Cellular proteins (25–30 μg/sample) were resolved by SDS–PAGE (4–20% gradient gel, BioRad), and transferred to Immobilon-P membranes (Millipore) followed by blocking with 5% BSA, incubation with primary antibody and detection of specific proteins by the enhanced chemiluminescence method according to the manufacturer's protocol (Amersham). Assessment of apoptosis-related proteolytic cleavage of poly(ADP-ribose) polymerase was performed as previously described (Soldatenkov *et al*, 1995) using a rabbit polyclonal antibody against the full length PARP (1 : 2000, Boehringer). For immunodetection of ubiquitinated proteins the cellular extracts were prepared in the presence of 0.1% SDS and 5 mM N-ethylmaleimide to inhibit potent isopeptidase activities that may affect the detection of multi-ubiquitinated proteins (Kim and Maniatis, 1996). The membrane-bound proteins were subjected to immunoblotting with monoclonal antisera to ubiquitin (Chemicon) at a dilution of 1 : 1000. Equal sample loading was confirmed by reprobing the same blots with a rabbit polyclonal antiserum against glyceraldehyde 3-phosphate dehydrogenase, G3PDH (1 : 5000, Trevigen).

Acknowledgements

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