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BUTYRATE INDUCED APOPTOSIS IN LYMPHOID CELLS PRECEDED BY TRANSIENT OVER-EXPRESSION OF HSP70 mRNA

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In addition to inducing differentiation in several cell types sodium butyrate is cytotoxic to lymphoid cells and causes apoptosis in HL-60 cells. We report here that butyrate treatment of the Burkitt's lymphoma cell line BL-30 causes cell death by apoptosis, as established by nuclear changes and DNA fragmentation. The kinetics of induction of apoptosis by butyrate revealed a considerably delayed response in comparison to that observed with heat treatment. At 4 h after heat (43.5°C) exposure, 50% of the cells were undergoing apoptosis whereas that level of apoptosis was only reached after 16 h of treatment with butyrate (5 mM). Apoptosis induced by both treatments was accompanied by a marked increase in hsp70 mRNA. The maximum response in mRNA preceded a rapid onset of apoptosis in both cases, and the response was transient. There was a corresponding increase in the level of hsp70 protein after exposure to both heat and butyrate which coincided with the pattern of increase in mRNA but protein levels remained high during the onset of apoptosis. The results obtained here provide further evidence for a relationship between differentiation and apoptosis.

Exposure of cells to sodium butyrate causes apoptosis (1,2), an inhibition of DNA synthesis and mitosis, alteration in cell morphology and a pattern of gene expression characteristic of differentiation (3,4). Early studies using Friend Erythroleukemic cells revealed that butyrate-induced differentiation was accompanied by the introduction of single strand breaks into DNA (5). It was suggested that this partial degradation of DNA was an early step in the control of differentiation. On the other hand inducers of differentiation such as butyrate were cytotoxic when used at similar concentrations in murine thymocytes and in the immature lymphoid-derived cell line CEM-C7 (6-7). The toxicity of butyrate was

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reduced in cells maintained under non-growth conditions. These early results supported a linkage between the processes of death and cell differentiation.

It is now increasingly clear that the processes of growth, differentiation and death are controlled by a complex series of steps in signal transduction pathways and overlap occurs between these pathways (8,9). Cell death is no longer regarded as a passive process but rather in the form of apoptosis or programmed cell death, is dependent on both pre-existing proteins and *de novo* protein synthesis (10-12). As shown with other human cells cycloheximide was incapable of inhibiting DNA fragmentation in a Burkitt's lymphoma cell line BM13674 (12). These data together with results with other inhibitors point to the importance of modification of existing proteins, such as by phosphorylation, in apoptosis (12,13).

Previous results from this laboratory have shown that both heat treatment and ionizing radiation induce apoptosis in the lymphoid cell lines CEM-C7 and BM13674 (12,13). In order to investigate further the relationship of cell differentiation to apoptosis we have compared the effects of butyrate and heat treatment on the Burkitts lymphoma cell line BL-30. Treatment with both agents gave rise to apoptosis, although the onset was considerably delayed after butyrate treatment. The induction of apoptosis was immediately preceded by a marked but transient increase in heat shock protein 70 (hsp70) mRNA in both cases. hsp70 also increased under these conditions and protein levels remained high at longer times after treatment.

Materials and Methods

Cell culture: The Burkitt's lymphoma cell line, BL-30 was used. Cell suspensions were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum and incubated in a 37°C incubator under a humidified 5% CO_2 environment. Cells were divided 15h prior to experimentation. Exponentially growing cells were used for all experiments at a density of $1x10^6$ cells/ml. Sodium butyrate was used at a final concentration of 5mM. This concentration was shown to be cytotoxic to BL-30 cells. Apoptosis was also induced in the cells using heat shock treatment at 43.5°C for 30 min followed by incubation of cells at 37°C.

Determination of cell viability: Cells numbers and viability were determined using light microscopy.

DNA-extraction and electrophoresis: Cells (10^7) were pelleted by centrifugation in a Beckman GS-6R centrifuge at 900 g for 5 min. The cells were lysed in 10ml of DNA lysis solution (1% SDS; 10mM Tris-HCl, pH 7.5; 1mM EDTA; 10mM NaCl) containing Proteinase K at a final concentration of 0.6 mg/ml and incubated overnight at 37°C. The solution was cooled, 2ml of saturated NaCl added and the sample mixed to precipitate protein and cell debris which were then pelleted by centrifugation at 5000 g for 15 min. High molecular weight DNA was precipitated with ethanol, washed with 70% ethanol and RNA was digested with RNAse (40 μg/ml). The DNA samples were electrophoresed on 2% agarose gels containing 1xTBE buffer at 10mA for approximately 2h. The gels were stained with ethidium bromide and the DNA visualised on a UV transilluminator.

hsp70 cDNA probe: An hsp70 cDNA sequence was isolated by subtractive hybridisation using cDNA prepared from untreated and heat/ionizing radiation treated BL-30 cells according to published protocols (14). Sequence analysis revealed that this cDNA contained the sequence corresponding to nucleotides 2010-2247 of the human hsp70 gene. The 0.25 kb PCR product was cloned into the EcoRI site of Bluescript.

Northern blot hybridization analysis: Poly(A)+ mRNA was extracted from untreated cells, at 0.5, 2 and 5 h post-heating incubation and at 8 and 12 h of incubation with 5mM sodium butyrate using affinity chromatography on oligo(dT) cellulose (15). mRNA samples (1 µg) were electrophoresed on 1% denaturing formaldehyde/agarose gels and transferred to Nylon membranes (Hybond-N+, Amersham). Radioactive human cDNA probe containing a fragment of hsp70 gene was used at a $0.5x10^6$ cpm/ml, prepared by the random primer labelling method with [α-^{32}P]-dCTP (16). After hybridization in 5xSSC; 5xDenhardt's solution; 0.02M NaH_2PO_4, 0.1% SDS at 65°C overnight, the filters were washed twice with 2xSSC-0.1% SDS for 10 min at room temperature, with 1xSSC-0.1% SDS for 15 min at 65°C, with 0.1 x SSC-0.1% SDS for 10 min at 65°C and exposed to x-ray film or to a Phosphorimager screen to quantify the blots.

Western blotting: For heat treatment cells were incubated for 0, 0.5, 2, 5 and 8 h and for butyrate 0, 4, 8 and 12 h. For immunoblotting, cells were washed in PBS and lysed by mild sonication (40 s) in lysis buffer (10 mM Tris-HC1 pH 7.4, 10 mM DTT, 1% SDS, 2mM PMSF and 20% glycerol). Proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose, blocked and incubated with hsp70 specific antibody. After incubation with secondary antibody (antimouse Ig alkaline phosphatase labelled), the hsp70 specific band was visualised using the substrates 5-bromo-1-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). After transfer the nitrocellulose membrane was stained with Ponceau red to determine protein loading. Quantitation was carried out using a Molecular Dynamics Densitometer.

Results

As observed previously with other B-cell lines exposure of BL-30 cells to 43.5°C for 30 min caused changes characteristic of apoptosis. A time-dependent increase in apoptotic cells, determined by light microscopy, occurred, reaching approximately 50% by 6h post-treatment (Fig. 1). When cells were incubated with sodium butyrate (5mM) only a small increase in the extent of apoptosis was observed up to 12h after which time there was a rapid increase in the percentage of apoptotic cells, reaching 80% by 24h (Fig. 1).

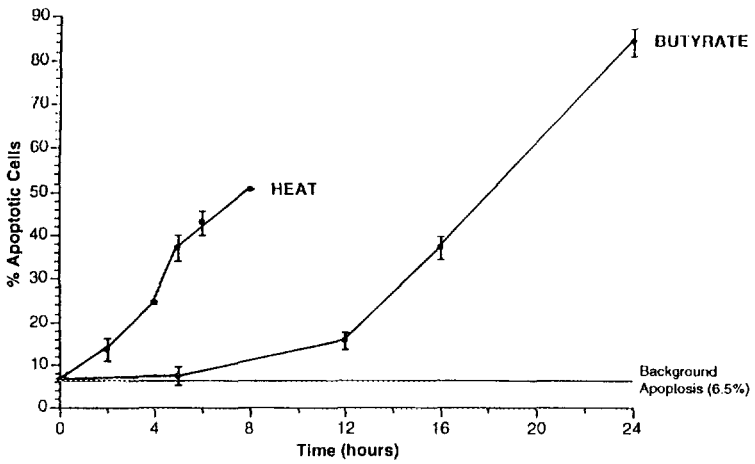

Figure 1. Time course of appearance of apoptosis in the Burkitt's lymphoma cell line BL-30 after exposure to either heat (43.5°C) for 30 min or continuous exposure to butyrate (5mM).

Further evidence that apoptosis was occurring was provided by agarose gel electrophoresis (Fig 2.) The characteristic DNA degradation into oligonucleosomal sized fragments was obtained after heat treatment (6h, lane 2) and with butyrate (16h, lane 4).

Since both heat shock and butyrate caused apoptosis in BL-30 cells and since both agents mediate their activity by altering gene expression it was of interest to identify common mRNAs overexpressed in response to these treatments which might be associated with apoptosis. Forty two clones were isolated from an irradiated/heat shock subtracted library prepared by the method of Wang and Brown (14). Northern analysis with 4 of these

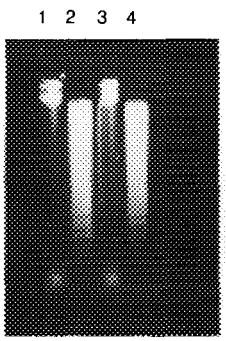

Figure 2. Pattern of DNA fragmentation after exposure of BL-30 cells to heat and butyrate. Lane 1, DNA from untreated cells; lane 2, DNA extracted from heat treated cells 6h post-heating; lane 3, DNA from untreated cells; lane 4, DNA extracted from cells exposed to 5mM butyrate for 16h. Electrophoresis was carried out on 2% agarose gels.

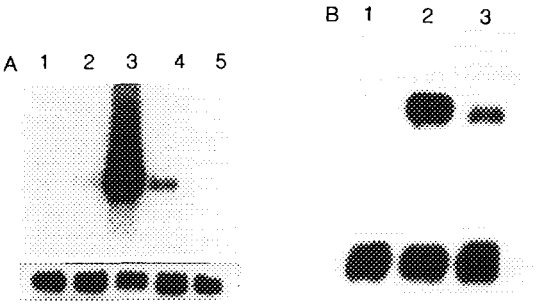

Figure 3. Northern blot analysis of polyA⁺RNA extracted from cells undergoing apoptosis after butyrate or heat treatment. A. Heat treatment. Lane 1, untreated; lane 2, 0.5h after heat; lane 3, 2h post-treatment; lane 4, 5h and lane 5, 7h. B. Butyrate treatment. Lane 1, untreated; lane 2, 8h incubation and lane 3, 12h incubation. The bottom panel is hybridization with β-globin to ensure that lane loadings are equal.

clones revealed a similar pattern of induction of an mRNA species of approximately the same size for heat and butyrate treated cells. No mRNA was detected in untreated cells but in heat treated cells mRNA was evident after 0.5h, reached a maximum at 2h and had disappeared by 7h post-treatment (Fig. 3A). The maximum steady state level was achieved just prior to the time when the extent of apoptosis increased rapidly (Fig. 1). A similar but delayed pattern of induction was evident after butyrate treatment. In this case the mRNA peaked at 8h, just prior to the onset of significant apoptosis (Fig. 3B).

DNA sequence analysis of these clones revealed 97% sequence identity to the major heat shock protein, hsp70 (results not shown). Use of an antibody specific for hsp70 demonstrated that there was a negligible amount of this protein in extracts from untreated cells but both heat and butyrate caused an increase in the protein. After heat treatment hsp70 increased markedly by 2h and this level was maintained up to 8h post-treatment (Fig. 4A). In the case of butyrate, hsp70 was detected at low levels by 4h and reached a

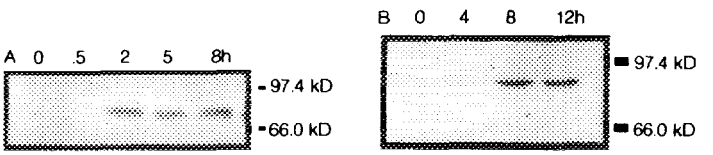

Figure 4. Western analysis of time course of appearance of hsp70 during apoptosis. A. Heat treated cells. B. Butyrate treated cells.

maximum by 12h incubation (Fig.4B). The increase in protein in both cases corresponded to the mRNA increase and preceded the appearance of significant levels of apoptosis.

Discussion

We have shown here that sodium butyrate, a compound characterized by its ability to induce cells to differentiate, is cytotoxic to the lymphoid cell line BL-30, and cell death is accomplished by apoptosis. These results point to common features in differentiation and apoptosis. The observation that inducers of differentiation in several cell types are cytotoxic to another lymphoid cell line CEM-C7 adds additional support to such a connection (7). In that study butyrate was shown to kill cells but it was not demonstrated that cytotoxicity was due to a mechanism involving apoptosis. Inducers of differentiation in Friend leukaemia cells (5) have been shown to induce cell death by apoptosis in unfractionated murine thymocytes (6). This was accompanied by a time-dependent degradation of chromatin into oligonucleosomal sized fragments, and inhibitors of protein synthesis prevented this process. A recent report has demonstrated that a variety of factors induce differentiation in mouse neuroblastoma cells and factor withdrawal can reverse this process leading to cell proliferation (17). Continous exposure of cells to differentiating agent without medium replacement led to marked cell death by apoptosis.

It is not evident at this stage what determines the fate of a particular cell type after exposure to a compound such as butyrate. There is some evidence that cells undergo differentiation initially and then die by apoptosis, but also data indicating that differentiation and apoptosis are not induced by the same signal (17, 18). It has been demonstrated that butyrate can affect specific transcription factors important for cell growth and differentiation at different levels of regulation. The protooncogene product c-Fos is initially induced by butyrate at a post-transcriptional level and at later times by induction of mRNA (19). Induction of differentiation in PC12 pheochromocytoma cells by butyrate led to an uncoupled overexpression of both c-fos and c-jun (3), but the butyrate-induced c-Fos protein was not

capable of negatively trans-regulating the c-fos promoter. These growth controlling factors are also upregulated in cells undergoing apoptosis. Prolonged activation of expression of c-jun has been reported in human fibroblasts treated with tumour necrosis factor α (20), in U-937 cells treated with 1-β-D arabinofuranosylcytosine (21), and in CEM-C7 cells exposed to dexamethosone or γ-radiation (Goldstone and Lavin - unpublished). A prolonged increase in c-fos mRNA has also be shown to be associated with apoptosis (22). Several other genes have been shown to increase in their expression during the process of apoptosis (23,24). Another regulatory gene c-myc has been reported to be transcriptionally downregulated after treatment of mouse plasmacytoma cells with butyrate (4). It is suggested that this is achieved by the prevention of access of a positive regulator to c-myc promoter elements or alternatively by the induction of a negative regulatory factor (25). Changes in expression of c-myc are also important in both cell proliferation and apoptosis. While activation of c-myc is most closely associated with cell proliferation, under conditions of growth factor withdrawal activation of c-myc causes some cells to undergo apoptosis (26,27). Antisense to c-myc prevents this form of activation-induced apoptosis. The decision to undergo cell death or differentiation in the presence of butyrate may be influenced by the relative levels of growth controlling factors such as c-Myc, c-Fos and c-Jun.

In general the heat shock response represents an adaptive process designed to increase cell survival. However, heat shock can also lead to apoptosis in mouse thymocytes (26), and in human lymphoid cell lines (12). We have shown here that a Burkitt's lymphoma cell line undergoes apoptosis in response to either heat or butyrate treatment. Common to both treatments was the transient appearance of hsp70 mRNA which peaked in both cases just prior to the onset of apoptosis. hsp70 protein levels also increased with similar kinetics but levels remained high as apoptosis progressed. The timing of this response could mean that hsp70 expression is designed to protect the cell but is countered by a series of other events forcing the cell towards the death pathway. Support for a role for hsp70 in

preventing apoptosis is provided by data with mouse thmocytes where incubation at 43°C for 20 min induced DNA fragmentation and cell death, but heat treatment was capable of decreasing apoptosis induced by dexamethasone (28). Thermotolerance and resistance to DNA fragmentation has also been reported for a human T lymphocyte cell line (PEER) exposed to 43°C for 30 min followed by incubation at 37°C (29). Contrary to the results reported here enhanced hsp70 synthesis correlated with the development of resistance to apoptosis in that study. Clearly, persistence of hsp70 is not protective against onset of apoptosis in Burkitt's cells.

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