Cytoplasmic acidification is not an effector mechanism of VP16 or DEX-induced apoptosis in CEM T leukaemia cells

R. S. P. Benson¹, C. Dive² and A. J. M. Watson¹, ⁺

¹Department of Medicine, University of Liverpool, UCD, The Duncan Building, Daulby Street, Liverpool, UK
²School of Biological Sciences, The University of Manchester, Stopford Building, Oxford Road, Manchester, UK

*Author for correspondence

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SUMMARY

The role of intracellular acidification in the execution phase of apoptosis is not well understood. Here we examine the effect of Bcl-2 over-expression on intracellular acidification occurring during apoptosis. We found, that in CEM cells, neither DEX nor VP16-induced apoptosis lead to a significant change in intracellular pH (pHi). Furthermore, we found that shifting pHᵢ away from physiological values was unable to induce chromatin condensation or poly(ADP-ribose) polymerase (PARP) cleavage in the presence of Bcl-2 over-expression. However, it was found that maximum chromatin condensation and PARP cleavage occurred at near physiological pHᵢ values. Taken together these data suggest that intracellular acidification does not trigger the effector phase of CEM apoptosis.

Key words: CEM cells, bcl-2, Death, Apoptosis, Acidification, Flow cytometry

INTRODUCTION

Apoptosis is a regulated cell death of importance in cell homeostasis, neoplasia and neurodegeneration (Arends and Wyllie, 1991). One of the central mediators of apoptosis is the caspase family (Cohen, 1997). Yet, it is still poorly understood how the actions of these proteases lead to the distinct morphology of apoptosis. Some investigators have proposed that intracellular acidification could be an important mediator for the development of apoptotic morphology. In particular, several of the candidate endonucleases, which when activated cause the classic non-random DNA cleavage observed in apoptotic nuclei, have maximum enzymatic activities at acidic pH values (Barry and Eastman, 1992, 1993; Gottlieb et al., 1995; Huang et al., 1995). Furthermore, many studies which have examined intracellular pH (pHi) have found that cells dying by apoptosis acidify (Benedetti et al., 1995; Huang et al., 1995; Li and Eastman, 1995; Rebollo et al., 1995; Gottlieb et al., 1996; Chen et al., 1997) and that Bcl-2 over-expression prevents both apoptosis and intracellular acidification (Meisenholder et al., 1996; Reynolds et al., 1996a). Our own studies suggest that changes in intracellular pH homeostasis form part of the anti-apoptotic action of v-Abl protein kinase (Chen et al., 1997). Moreover, in some systems, prevention of cellular acidification abrogated subsequent apoptosis (Pérez-Sala et al., 1995; Gottlieb et al., 1996) while in another system artificially lowering pHᵢ lead to apoptosis (Furlong et al., 1997). While these studies implicate pHi as a mediator of apoptosis, the finding that pHᵢ decreases with apoptotic death is not universal, with some studies finding no change in pHᵢ (Kluck et al., 1993; Whyte et al., 1993; Furuya et al., 1994) while others find an alkalinization (Zhu and Loh, 1995; Tsao and Lei, 1996; Dai et al., 1998). Similarly, in cytotoxic T lymphocytes, which undergo apoptosis and acidification upon IL-2 withdrawal, prevention of this acidification by incubating the cells in an alkaline medium, did not abrogate apoptosis as measured by DNA digestion (Li and Eastman, 1995). Finally, two recent studies found that low extracellular pH inhibited DNA breakage (Lee et al., 1997) and caspase activity (Reynolds et al., 1997).

A key issue when studying pHᵢ change in apoptosis, is whether this change is occurring as a secondary consequence of or is an important regulator of the execution of the cell death programme. This question is becoming ever more important given recent evidence that the Bcl-2 family members could be behaving as pores which have ion conductances that are sensitive to pH (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). In order to resolve this issue we have demonstrated the successful manipulation of pHi in the presence of Bcl-2 over-expression. However, it was found that maximum chromatin condensation and PARP cleavage occurred at near physiological pHᵢ values. Taken together these data suggest that intracellular acidification does not trigger the effector phase of CEM apoptosis.

MATERIALS AND METHODS

Materials

Unless otherwise stated all culture media and supplements were purchased from Life Technologies (Paisley, Scotland, UK), fluorescent probes from Molecular Probes Inc. (Eugene, OR) and all other materials from Sigma Ltd (Poole, UK).

CEMneo and CEMBcl-2 cells, stably transfected with the retro-viral
vectors pZipNeo and pZipBcl-2, respectively, were kindly provided by Dr Seamus Martin (La Jolla Institute for Allergy and Immunology, La Jolla CA, USA). Experiments using the cell lines were performed on cells of passage number 10 to 50. Cells were maintained in a 5% CO2 humidified incubator at 37°C in RPMI 1640 medium supplemented with 10 µg penicillin, 50 µg streptomycin per 500 ml, 1% glutamine and 10% foetal bovine serum (FBS). Cells were routinely seeded at a density of 1x10^5 cells/ml and passaged every 3 or 4 days. During experiments, cells in log phase (2x10^5/ml-5x10^5/ml) were exposed to 5 µM dexamethasone (DEX) or 5 µM etoposide (VP16). Control cultures received either ethanol (for DEX) or DMSO (for VP16) vehicle at 0.1%, v/v.

Fluorescence microscopy
The nuclear morphology of CEM cells was examined after staining with acridine orange (AO, 5 µg/ml) using an Olympus BH2 RFCA fluorescence microscope as previously described (Wood et al., 1994). Apoptotic cells were classified as those with condensed and fragmented chromatin.

Measurement of intracellular pH (pHi) using fluorescence spectrophotometry
Cells were incubated directly with BCECF (final concentration 500 nM) for 20 minutes at 37°C, washed in isotonic phosphate buffered saline (PBS). Samples were excited at 440 and 500 nm and the emission fluorescence measured at 530 nm and averaged over a 3 minute period. During this time the measured fluorescent ratio was found to be stable. Calibration was achieved by lysing the cells with Triton X-100 and decreasing the extracellular pH, using small volumes of concentrated (1 M) HCl, while measuring with a pH sensitive probe. A correction for extracellular calibration was applied as previously described (Lau et al., 1989).

Measurement of intracellular pH (pHi) using multiparameter flow cytometry
We have shown previously that CEM cells with condensed chromatin fluoresce more intensely when loaded for short periods at 25°C with Hoechst 33342 (Ho342) (Benson et al., 1996). Using cells which were dual labelled with SNARF and Ho342, we were able to measure simultaneously chromatin condensation and pHi using a method similar to one previously described (Reynolds et al., 1996b). In brief, SNARF and Ho342 were added to the cell suspension to give final concentrations of 2.2 µM and 10 µM, respectively. The cells were incubated at 37°C for 15 minutes with SNARF and then washed in PBS at room temperature. Five minutes prior to analysis Ho342 was added to the cell suspension and ten thousand cells were analysed, at a flow rate of 300–400 cells/second, on a FACS Vantage flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an Enterprise laser (Innova Technology, Coherent Inc., Palo Alto, CA). Excitation of SNARF was at 488 nm and Ho342 at 351-364 nm. The ratio of SNARF fluorescent emissions at 630 nm and 575 nm was calculated together with the blue (424 nm) Ho342 fluorescence. Comparison of single and dual labelled calibration experiments confirmed that neither SNARF nor Ho342 interfered with each other except at very alkaline (above 8) pH values.

pHi clamp experiments
CEMneo and CEMBcl-2 cells were treated with VP16 (5 µM) for 4 hours. The cells were then transferred to a high (120 mM) K+ medium with a set pH between 6.5 and 8 (rising in increments of 0.2 pH units). Nigericin (10 µM) and VP16 (5 µM) were added and the cells were further incubated for 4 hours. The nuclear morphology of the cells was then assessed as described above with all samples coded to prevent experimenter bias, or the cells were prepared for immunoblotting as described below.

PARP cleavage
For each sample a lysate was prepared by sonicating cells in a lysing buffer (Chen et al., 1997) and 20 µg of protein separated by SDS-PAGE (7.5% resolving gel, 4.5% stacking gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. The blot was blocked with 5% dried milk in PBS and then incubated for 2 hours at 23°C with the primary antibody, murine anti-PARP, kindly donated by Drs S. Aoufouchi and C. Milstein, MRC Centre, Cambridge UK). The immuno-reactive proteins were visualised using rabbit horse radish peroxidase conjugated anti-mouse-IgG (1:1000) and enhanced chemiluminescence (ECL).

Measurement of v/v of using JC-1
Cell cultures between 2-8x10^5 cells/ml were incubated for 10 minutes at 37°C with JC-1 at a final dye concentration of 10 µg/ml. The cells were then washed once and resuspended in PBS. Analysis was performed on a FACS Vantage flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an Enterprise laser (Innova Technology, Coherent Inc., Palo Alto, CA) using a method recently described (Brunet et al., 1997).

RESULTS
The effect of Bcl-2 over-expression on chromatin condensation and pHi
As shown in Table 1, treatment of CEMneo cells with either DEX or VP16 leads to apoptosis as judged by nuclear morphology. Following exposure to DEX or VP16 there was a lag phase of 24 or 4 hours, respectively, in which no morphological changes could be detected. Bcl-2 over-expression completely inhibited these nuclear changes at the time points examined.

To examine whether Bcl-2 prevented intracellular acidification during the onset of apoptosis, the mean pHi of CEMneo and CEMBcl-2 cell populations was measured using both fluorescence spectroscopy and flow cytometry. We were not able to detect a significant decrease in mean pHi in cells treated with either DEX (Fig. 1A) or VP16 (Fig. 1B), furthermore, Bcl-2 over-expression did not significantly alter the mean pHi in either treatment condition.

One possible limitation with this method is that fluorescent spectrophotometry can only measure the mean fluorescent ratio of the whole cell population. If the cytosol of a small subset of apoptotic cells had become acidic, this may not be resolved by

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Neo Control</th>
<th>Neo Treated</th>
<th>Bcl-2 Control</th>
<th>Bcl-2</th>
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<tr>
<td>24</td>
<td>1.1±0.5</td>
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<td>6.0±7</td>
<td>1.7±0.5</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>96</td>
<td>1.4±0.1</td>
<td>75±8</td>
<td>2.9±0.6</td>
<td>1±1</td>
</tr>
</tbody>
</table>

Time course for chromatin condensation in CEMneo (Neo) and CEMBcl-2 (Bcl-2) cells treated for up to 96 hours with 5 µM DEX (upper section) or 5 mM VP16 (lower section). Vehicle controls received 0.1%, v/v, ethanol or DMSO for DEX and VP16, respectively. Chromatin condensation was assessed using the nuclear dye AO and is expressed as the percentage of cells which appeared to have a condensed nucleus under the microscope. Each data point represents the mean ± s.e.m. of at least 4 experiments.
Intracellular acidification does not defeat the anti-apoptotic effect of Bcl-2

While we are unable to detect pHi changes in CEM cells undergoing DEX- or VP16-induced apoptosis, there is still the possibility that pHi changes occur in cells which are no longer able to load the SNARF pHi indicator. Such changes, if they do occur, will be after the increase in Ho342 fluorescence, as many cells in this population are still able to load SNARF. It is therefore possible that late changes in pHi might be important for chromatin condensation in DEX and/or VP16-induced apoptosis in CEM cells. To exclude this possibility, we determined whether it is possible to defeat the protective effects of Bcl-2 over-expression by directly manipulating pHi. Fig. 3A shows typical fluorescence micrographs of CEMneo and CEMBcl-2 cells treated for 4 hours with VP16 and then further subjected to 4 hours pHi clamping using a high K+ extracellular solution and ionophore nigericin (Watson et al., 1991). The pH units shown in Fig. 3A correspond to the extracellular pH of the incubation medium. It can be clearly seen that manipulation of media pH from 6.8 through to 8 did not defeat the anti-apoptotic effect of Bcl-2 over-expression. More surprisingly, large extracellular perturbations from physiological pH resulted in the suppression of chromatin condensation in CEMneo cells (Fig. 3A).

In order to test that pHi was being altered by the nigericin clamp, cells which had been subjected to this methodology were loaded with the pH fluorescent indicator BCECF. The pHi was measured on a fluorescence spectrophotometer and the cells were then lysed using Triton X-100. This procedure released the dye into the extracellular medium so that the resulting fluorescence ratio signal then corresponded to the extracellular or medium pH. Using this protocol, it was possible to construct a calibration curve for medium pH to pHi. The calibration curve is shown in Fig. 3B. This figure demonstrates that the nigericin clamp was effective albeit that the magnitude of pHi changes were attenuated compared to the shift in buffer pH.

One potential complication with using nigericin to manipulate pHi is that nigericin also affects intracellular membranes and the ATPase activity in the mitochondria (Rottenberg and Scarpa, 1974). To eliminate the possibility that the suppression of apoptosis at non-physiological pHi was due to indirect effects of nigericin on mitochondrial membrane potential (\(\psi_M\)), we measured \(\psi_M\) at the various pHi values. As expected the maximum number of cells with depolarized mitochondria occurred in CEMneo cells incubated at extracellular pH of 7.2-7.4. More importantly, this depolarisation was not observed in CEMBcl-2 cells at the same external pH values (percentage of cells with normal \(\psi_M\) at medium pH of 7.2 were 54% versus 92% for CEMneo and CEMBcl-2 cells, respectively) which demonstrates that the loss in \(\psi_M\) is due to apoptosis and not an indirect effect of nigericin. Furthermore, no hyperpolarisation was observed in any of the treatment conditions. Similarly, the protonophore carbonyl cyanide m-cholorophenylhydrazone (mCCCP; 100 \(\mu\)M) did not induce apoptosis when incubated with CEM-C7A cells for 24 hours (AO positive in mCCCP treated cells was never greater than 5%) even though it did depolarise the mitochondria. Likewise co-incubation of CEMneo cells with nigericin and mCCCP did not significantly alter the amount of apoptosis observed with nigericin alone (45±8 vs 49±10% for nigericin and nigericin + mCCCP, respectively).

Fig. 3C shows the statistical summaries for the number of
cells with condensed nuclear chromatin at various pH. In this figure the buffer pH has been replaced by the measured pH using the calibration curve of Fig. 3B. It is clear from this figure that the protective effects of Bcl-2 are not defeated by either lower or raising pH. Furthermore, moving pH from physiological values results in the suppression of chromatin condensation as judged by AO staining of the nucleus.

One possible explanation for the suppression of apoptosis in CEMneo cells when pH is moved away from normal physiological values could be that caspases require a physiologically normal pH in order to function. In order to test this we examined the effect of pH on PARP cleavage (a target for caspase-3) in CEMneo and CEMBcl-2 cells. As shown in Fig. 4, the kinetics of PARP cleavage closely mirrored the nuclear changes observed in Fig. 3, with maximum cleavage observed at pH 7.5 in CEMneo cells. PARP cleavage decreased with excursions of pH away from this value. While a low level of PARP cleavage, which was above the untreated control, was consistently observed in CEMBcl-2 cells at all pH this level was never significantly altered by varying pH and was always well below the treated CEMneo control.

DISCUSSION

In CEM cells, neither DEX nor VP16-induced apoptosis resulted in changes in pH. However, these data are in contrast to experiments by Meisenholder et al., who found that Fas-induced apoptosis in CEM cells caused intracellular acidification which was prevented by both caspase inhibition and Bcl-2 over-expression (Meisenholder et al., 1996). These data suggest, that in Fas-induced apoptosis intracellular pH is a mediator of apoptosis that acts after caspase activation and Bcl-2 sensitive events. These conflicting results are not explained by differences in methodology as we are able to detect acidification during apoptosis in other cell lines (Chen et al., 1997). To exclude the possibility that there were functionally important pH changes which we could not detect, we examined whether we could re-establish VP16-induced apoptosis in CEM cells over-expressing Bcl-2 by directly manipulating intracellular pH. We found that in CEM cells treated with VP16, directly manipulating pH did not defeat the protective effects of Bcl-2. Crucially, movement of pH away from physiological values actually suppressed the nuclear changes of apoptosis and this may have been partly due to the suppression of the death caspases especially caspase 3. Recently, the pKa values for the known caspases have been measured. In vitro enzymatic studies show that caspase 3 has a maximum activity at pH 7.5 (N. Thornberry, personal communication). This is in good agreement with our data that shows maximum PARP cleavage and chromatin condensation at pH of 7.5. The suppression of caspase-3 by acidic pH has also been demonstrated in cell free systems (Dubrez et al., 1996).
Cytoplasmic acidification in CEM cell apoptosis

Furthermore, many cells possess a range of endonucleases which have maximum activities in acidic (Barry and Eastman, 1992, 1993; Gottlieb et al., 1995; Huang et al., 1995) and alkaline (Ribeiro and Carson, 1993; Matsubara et al., 1994; Shiokawa et al., 1994) pH environments. The presence of both endonucleases and caspases that work in various pHi environments, may be so that efficient cellular destruction pathway can proceed even when the control mechanisms for pHi homeostasis have collapsed. If this hypothesis is correct, then the suppression of apoptosis in CEM cells when pHi is moved from normal physiological values could be due to a low expression of caspases and/or endonucleases which function at non-physiological pH values. The inverse of such an argument may be evoked to explain why some studies appear to find pHi changes are important in apoptosis. The cells used in such range (N. Thornberry, personal communication). Furthermore, many cells possess a range of endonucleases which have maximum activities in acidic (Barry and Eastman, 1992, 1993; Gottlieb et al., 1995; Huang et al., 1995) and alkaline (Ribeiro and Carson, 1993; Matsubara et al., 1994; Shiokawa et al., 1994) pH environments. The presence of both endonucleases

Fig. 3. (A) Fluorescent micrographs showing the effect of buffer pH on chromatin condensation in CEMneo (top row; 1-3) or CEM_BC-2 (bottom row; 4-6) cells treated with VP16 for 8 hours. After 4 hours of initial treatment they were transferred to a high K+ buffer (120 mM) and subjected to a range of extracellular pH values (6.8-8.0) in the presence of nigericin. Cells were then stained with the nuclear dye AO (5 µg/ml) as described in Materials and Methods. Note that maximum apoptosis occurs at an extracellular pH of 7.4 (2). The buffer pH for each micrograph is indicated on the figure. (B) Comparison of pHi with buffer pH after 4 hours of nigericin treatment. Treated cells were loaded with BCECF (100 nM) as described in Materials and Methods. The pHi was determined by spectrophotometry and the cells were then lysed with a small volume of 10% Triton to calibrate the BCECF fluorescent ratio signals with the external buffer pH. Each data point represents the mean ± s.e.m. of three experiments. (C) Bar chart showing the effect of pHi on chromatin condensation in CEMneo (light grey bars) and CEM_BC-2 (dark grey bars) cells. The abscissa now shows the intracellular pH as determined in B. The ordinate is the percentage of cells with condensed chromatin as judged by morphological examination (see A). Over 150 cells were counted in each experiment and each bar represents the mean ± s.e.m. of 6 experiments.

Fig. 4. The effect of pHi on PARP cleavage. Cell lysates were prepared from CEMneo and CEM_BC-2 cells treated as described in Fig. 3A. Immunoblots for the presence of PARP and its cleaved product were then carried out as described in the methods. PARP and its cleavage product are indicated on the figure as well as the pHi for each cell lysate. Lanes marked with a ‘+’ indicate that the lysate was from the CEM_BC-2 cell line while lanes marked with a ‘-’ are CEMneo lysates. The two far right lanes are control lysates. The positive control (marked +ve) are CEMneo cell lysates treated with VP16 for 16 hours. The negative control (marked −ve) are lysates from untreated CEMneo cells. The gel represents 4 other experiments.

<table>
<thead>
<tr>
<th>pHi</th>
<th>6.6</th>
<th>7.0</th>
<th>7.1</th>
<th>7.5</th>
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<tbody>
<tr>
<td>PARP</td>
<td></td>
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</tr>
<tr>
<td>Bcl-2</td>
<td>−</td>
<td>−</td>
<td>+</td>
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Controls
studies may have an expression profile of caspases and/or endonucleases which function at a more acidic pH. However, in these systems intracellular acidification is still only permissive for apoptosis and not a causative factor for commitment to the death process.

In summary, we have investigated the role of acidification in CEM apoptosis. We find that in the CEM model, intracellular acidification is not a universal or necessary feature of apoptosis. Rather, maintenance of a relatively normal pH is required for the maximum activity of caspase 3 and for efficient DEX- and VP16-induced apoptosis in CEM cells.

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