Effect of anti-apoptotic genes and peptide inhibitors on cytoplasmic acidification during apoptosis

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Abstract Cytoplasmic acidification has been shown to occur during the apoptotic process of cell death although its relation with other events in the process are not yet clear. AK-5 tumor cells have been shown to undergo apoptosis upon treatment with stimuli like dexamethasone (1 μM) or with serum from animals that reject AK-5 tumor. The current study was designed to measure the extent of cytoplasmic acidification during apoptosis in AK-5 cells and to study the effect of antiapopoptic genes and peptide inhibitors on cytoplasmic acidification. Our results show that AK-5 cells when triggered into apoptosis show intracellular acidification by about 0.2 pH units and this is prevented when cells are treated with peptide inhibitors. In addition cytoplasmic acidification does not occur when AK-5 cells are transfected with anti-apoptotic genes Nedd-2 A.S, Crm A or bcl-2 which inhibit apoptosis.

Key words: Tumor cell apoptosis; pH reduction; Anti-apoptotic gene; Peptide inhibitor

1. Introduction

Apoptosis is now a well recognized process of cell death which occurs mainly under physiological conditions wherein the cell actively participates in its own death. The detailed mechanism of induction of apoptosis or the nature of the signalling molecules that transmit the apoptotic signal into the nucleus is not yet well understood. However, the execution phase of the apoptotic program is better understood and several cysteine proteinases [1,2] and specific endonucleases [3] have been implicated in this phase. One of the endonucleases involved in apoptosis has been shown to be activated by lowering of intracellular pH $[pH]_i$ [4]. Recently the intracellular acidification has been shown to result from a change in the setpoint of the Na^+/H^+-antiport which may result from its dephosphorylation [5].

The importance of proteolysis in apoptosis is well established from the genetic analysis in *C. elegans* [6]. Similarly the mammalian homologs of the *ced* family of genes which participate in apoptosis are also known [1,2,7]. *Bcl-2*, which is an anti-apoptotic gene similar to *ced-9* inhibits the cysteine protease activity [8] and has also been shown to act at a point upstream of protease activation and acidification [9]. Thus intracellular acidification is considered to be an essential event in the apoptotic program of a cell.

We have been studying the mechanisms involved in the spontaneous regression of a rat histiocytoma, AK-5 [10], and have demonstrated the participation of natural killer (NK) cells in antibody-dependent cellular cytotoxicity (ADCC) [11]. AK-5 cells are killed by NK cells through necrosis and apoptosis [12] both in vitro [13] and in vivo [14]. Cysteine proteinases like interleukin-1β converting enzyme (ICE) (caspase-1), YAMA/CPP32 (caspase-3) and Nedd-2 (caspase-2) participate in the execution phase of apoptosis in AK-5 cells and *bcl-2* gene product inhibits the apoptosis completely (unpublished observations). In the present investigation we have measured the extent of intracellular acidification while the tumor cell is undergoing apoptosis, using a fluorescent probe 2′,7′-bis-(-2 carboxyethyl)-5,6 carboxy-fluorescein acetomethoxy ester (BCECF-AM). We also studied the pH changes in the presence of cysteine protease inhibitors (like YVAD, ester DEVD) and CrmA. Similarly intracellular acidification induced by apoptotic stimuli in AK-5 cells transfected with the *bcl-2* gene was also studied. Results presented suggest a correlation of intracellular acidification with apoptosis, as change in $[pH]_i$ was not seen in the presence of inhibitors of apoptosis.

2. Materials and methods

2.1. Tumor and cell line

AK-5 tumor which is maintained as ascites was adapted to grow for several generations in DMEM with 10% FCS in the presence of penicillin (100 U/ml) and streptomycin (50 μg/ml). A single cell clone of AK-5 called BC-8 has been used in these studies to avoid ambiguity in results due to tumor heterogeneity [15].

2.2. Apoptosis assay and isolation of serum factor

BC-8 cells (1×10^6) were incubated with serum (45% ammonium sulfate fraction of anti-AK-5 serum) (10%) or dexamethasone (DEX, 1 μM) for different time periods. The cells were washed, fixed in ethanol and stained with propidium iodide (PI) and analyzed by flow cytometry.

For isolating the serum factor, serum from rats that had rejected the solid tumor after rechallenging with 5×10^6 AK-5 cells intraperitoneally, was collected. The sera from different animals were tested for the presence of anti-AK-5 antibody by their ability to lyse AK-5 cells through complement fixation and positive samples were pooled. Ten milliliters of anti-AK-5 serum was diluted with phosphate-buffered saline (PBS), 22.5 g of ammonium sulfate was added, volume made up to 50 ml (to give 45% ammonium sulfate fraction) and was kept at 4°C for 4 h with constant stirring. At the end of 4 h, the serum was spun at $12\,000\times g$ in HB-4 rotor for 20 min. The precipitate was washed, dissolved in 2 ml of cold PBS and dialysed overnight with three changes of PBS at 4°C to remove traces of ammonium sulfate in the sample. This is referred to as serum factor.

2.3. Propidium iodide staining and flow cytometry

BC-8 cells after the apoptosis assay were washed with PBS, and fixed in 70% ethanol. Tumor cells were stained with PI reagent (Calbiochem, 50 μg/ml in 0.1% sodium citrate containing 0.1% Triton X-100) and analyzed under fluorescent microscope.

2.4. DNA extraction and electrophoresis

Fixed tumor cells were washed, suspended in citrate-phosphate buffer and the fragmented DNA was extracted following the previ-

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ously published procedure [16]. Fragmented DNA with loading buffer was electrophoresed on 0.86% agarose gel at 2 V/cm for 16 h. DNA in the gels was visualized under UV light after staining with 5 μg/ml ethidium bromide.

2.5. DNA transfections and cloning

BC-8 cells (2×10^6) were transfected with 5 μg of the linearized plasmids, CrmA [7], Nedd-2 A.S [17] and bcl-2 [18] by electroporation method as described earlier [19]. Geneticin (G418) was added (400 μg/ml) and the cells were selected for 14 days. Bcl-2 transfected clones were selected with hygromycin B (400 μg/ml) for 14 days. Single cell clones were obtained by limiting dilution procedure. Clones were screened by Southern and Northern hybridizations and the positive clones were expanded and used in the studies.

2.6. Treatment with protease inhibitors

BC-8 cells and the transfected clones were pre-incubated with cysteine protease inhibitors Ac-YVAD-cmk and Ac-DEVD-CHO (Bachem, Switzerland) for 30 min prior to their treatment with the inducers of apoptosis. After a preliminary titration of the peptide inhibitors, YVAD and DEVD were used at a concentration of 50 μM and 400 μM, respectively. Control cells were treated with appropriate concentrations of DMSO or ethanol the solvent in which the peptide inhibitors were dissolved. These cells were processed for pH measurements.

2.7. Measurement of intracellular pH

Change in $[pH]_i$ was measured using BCECF-AM as described earlier [20]. Specifically BC-8 cells and the transfected clones (2×10^6 cells in 2 ml) were treated with BCECF-AM at a final concentration of 1 μM. After 30 min of incubation with BCECF-AM, cells were washed to remove external probe resuspended in DMEM-FCS and treated with apoptotic stimuli, DEX (1 μM) or serum factor (10%) or with peptide inhibitors for different periods of times. At the end of the treatment cells were washed and resuspended in PBS-MOPS and used for fluorescence measurements.

Preliminary observations indicated 90 and 180 min incubation as the optimal time for the induction of apoptosis related pH changes induced by DEX and serum factor, respectively. It is to be noted that there is no change in the maximum fluorescence intensity observed in a particular sample over a period of time. Therefore the lowering of fluorescence intensity after the addition of DEX or serum factor most probably reflects intracellular acidification. Standard curve for the pH-dependent fluorescence of BCECF was generated as described earlier [20]. Briefly 2×10^7 cells were loaded with the fluorescent probe for 30 min, the cells were washed, resuspended in 20 ml of PBS-MOPS containing 0.1% Triton X-100 and homogenized. The homogenate was centrifuged at 10000 rpm in HB-4 rotor ($15000\times g$) to remove the cell debris and pH of 3 ml volumes of it was adjusted to different values between 6 and 8. Standard curve was generated after recording the fluorescence spectra and peak intensity at 530 nM of the cell lysates at different pH values.

3. Results

3.1. Induction of apoptosis

AK-5/BC-8 tumor cells undergo apoptosis when treated with either DEX or with the serum of tumor-bearing animals. However, the identity of the serum factor that induces apoptosis is not yet clear. Tumor cells show condensation of chromatin and formation of apoptotic bodies in the presence of serum factor (Fig. 1). Similarly when BC-8 cells are treated with DEX, there is fragmentation of cellular DNA which is typical of apoptotic cells (Fig. 2). Apoptosis of AK-5 cells plays an important role in the death of tumor cells leading to spontaneous regression of the tumor [14]. We have also demonstrated the participation of cysteine proteinases ICE, CPP32/YAMA and Nedd-2 in the execution of apoptosis in BC-8 cells (unpublished observations).

Table 1
pH changes in BC-8 cells after induction of apoptosis[a]

Group	Incubation time(min)	pH
Control	90	6.63
Control	180	6.12
+DEX	90	6.44 (0.19)
+Serum factor	180	6.32 (0.20)
+DEX+YVAD	90	6.54 (0.09)
+Serum factor+YVAD	180	6.15 (0.03)
+DEX+DEVD	90	6.63 (0.00)
+Serum factor+DEVD	180	6.47 (0.35)[b]

[a]BC-8 cells (5×10^6) were incubated with BCECF-AM, washed and treated with either DEX or the serum factor. 2×10^6 cells were washed, suspended in PBS and the fluorescence spectra were recorded. pH units were calculated from the standard curve generated for the dye at different pH values. The values shown are representative of 5 similar experiments

[b]This increase in pH by 0.35 U as compared to the control cannot be explained as of now.

3.2. Changes in intracellular pH after induction of apoptosis

Changes in the internal pH of the cells $[pH]_i$ was measured using a pH-dependent fluorescent probe BCECF-AM. Fig. 3 shows the corrected fluorescence spectra of the dye taken up by the BC-8 cells and after treatment with the apoptotic inducers, DEX (Fig. 3A) and the serum factor (Fig. 3B). As can be seen there is a reduction in fluorescence intensity of BCECF taken up by cells in the presence of DEX and serum factor. The corrected spectra presented here are free from influences due to wavelength characteristic of the analysis system. Fluorescence spectra of BCECF in the cell lysates adjusted to different pH values were recorded and a standard curve as generated by plotting fluorescence intensity versus pH. DEX, which is an inducer of apoptosis in BC-8 cells shows the lowering of pH by 0.19 U as compared to the appropriate control (Fig. 3A, spectra a and b and Table 1).

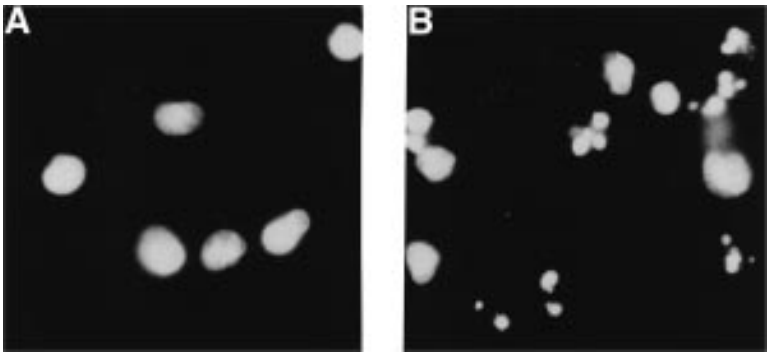

Fig. 1. AK-5 tumor cells treated with serum factor. A: Control. B: Serum-treated cells (notice the formation of apoptotic bodies when treated with serum factor).

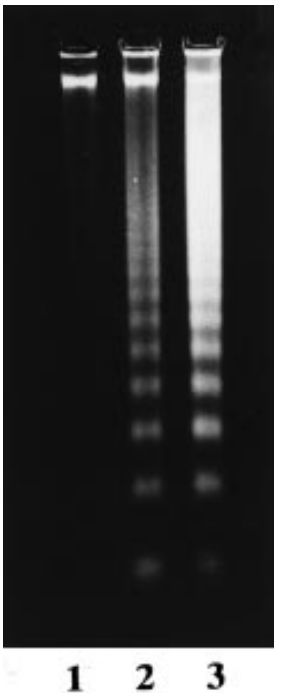

Fig. 2. Induction of DNA fragmentation in AK-5 cells after treatment with dexamethasone for 16 h. Lanes 1, control; lanes 2 and 3, dexamethasone-treated cells.

Similarly, serum factor induced a pH change of 0.20 U (Fig. 3B, spectra c and d and Table 1). There was no lowering of $[pH]_i$ or the acidification was less in the presence of inhibitors of apoptosis DEVD and YVAD in BC-8 cells (Table 1).

3.3. Inhibition of apoptosis and intracellular acidification

In order to confirm that the pH changes observed in BC-8 cells were due to the induction of apoptosis induced by DEX or serum factor, we introduced inhibitors of cysteine proteinase genes Nedd-2 A.S, CrmA or Bcl-2 into BC-8 tumor cells by DNA transfection procedures. Nedd-2 in antisense orientation (Nedd-2 A.S) inhibits Nedd-2 protease [17], similarly CrmA is a specific inhibitor for ICE protease [7] and bcl-2 has been shown to act upstream of cysteine proteases and thus acts as a strong inhibitor of apoptosis [18]. There was no significant change in the internal pH in all the three transfected clones which expressed inhibitors of proteases in the presence of DEX or serum factor (Table 2). These results confirm our earlier observations that pH change of the order of about 0.2 U corresponds to the apoptotic process initiated by either DEX or serum in these cells.

Table 2
Apoptosis related pH changes in BC-8 cells and the transfected clones[a]

Group	pH		
	Control	DEX	Serum factor
BC-8	6.63	6.44 (0.19)	6.52 (0.11)
Nedd-A.S	6.50	6.48 (0.02)	n.d.
CrmA	6.50	6.47 (0.03)	n.d.
Bcl-2	6.45	6.49 (0.04)	6.39 (0.06)

[a]BC-8 control cells and the transfected clones were incubated with BCECF-AM and induced for apoptosis with DEX or the serum factor. The values shown are representative of 3 similar experiments. n.d. denotes not determined.

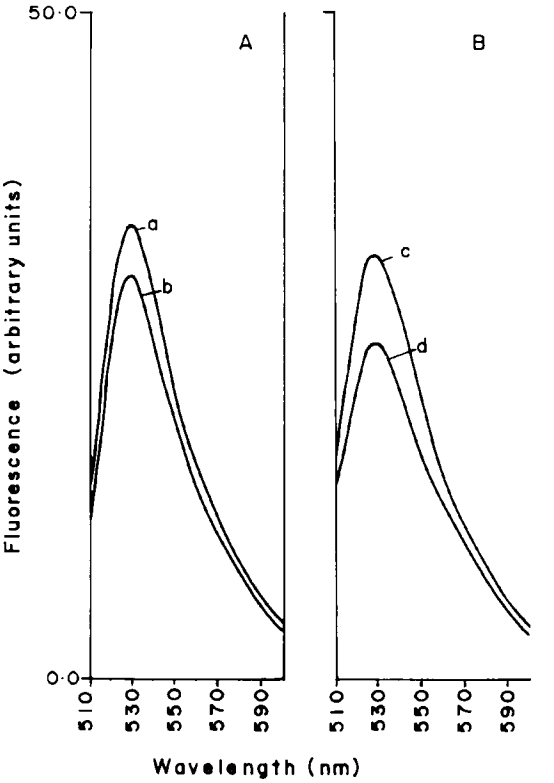

Fig. 3. Corrected spectra of BCECF-AM taken up by AK-5 cells treated with inducers of apoptosis, dexamethasone or serum factor. AK-5 cells were incubated with BCECF-AM for 30 min in DMEM buffered with MOPS, washed and resuspended in DMEM-MOPS in the presence and absence of dexamethasone (1 μM) for 90 min or serum factor (10%) for 180 min as described in Section 2. Cells were pelleted, washed and resuspended in PBS-MOPS and the spectra of the cells were recorded. Keeping the excitation wavelength at 500 nm and excitation and emission bandpass at 3 nm each. (A) a, control; b, dexamethasone for 90 min. (B) c, control; d, serum factor for 180 min.

4. Discussion

Acidification of cells undergoing apoptosis is a well recognized feature established in a variety of systems [5,9,21]; however its relation to other events in the apoptotic process are not known. It is also thought that the cytoplasmic acidification may be an irreversible event, which may be serving as a global switch to inactivate normal cellular processes operating at neutral pH. HL-60 and ML-1 cells when induced into apoptotic program with anticancer drug, etoposide, showed a discrete population of cells with a pH of about 0.7 U below that of control cells [22].

Cells have been shown to regulate their pH through a variety of ion transport mechanisms, including Na^+/H^+-antiports [5]. A wide variety of external signals including growth factors have been shown to enhance the affinity of the antiport for H^+ which is also dependent on phosphorylation [23].

Apoptosis or programmed cell death is a physiological process during development, tissue remodelling and cell turnover [24]. Regulation of apoptosis plays a crucial role in many pathological conditions including cancer, AIDS and neurode-

generation [25]. The importance of understanding the mechanisms of induction of apoptosis in tumor cells is enormous for cancer therapy. Recent genetic and biochemical studies have suggested that proteases and endonucleases play a prominent role in the execution phase of the apoptotic cell death. We have also evidence to suggest the participation of ICE-like proteases in the apoptosis of AK-5 cells (unpublished observations). Apoptosis in AK-5 cell is mediated by activated NK cells and another as yet unidentified factor present in the serum of AK-5 tumor rejecting animals. In addition, DEX also induces apoptosis in AK-5 cells. Introduction of CrmA (inhibitor for ICE protease) and Nedd-2 in antisense orientation inhibited the apoptotic activity of AK-5 cells. Similarly peptide inhibitors YVAD and DEVD also inhibited apoptosis in AK-5 cells.

In the present study we have investigated the process of the cytoplasmic acidification in AK-5 cells undergoing apoptosis. There is a 0.2 U change in the $[pH]_i$ of AK-5 cells which were triggered into apoptosis with either DEX or serum factor (Table 1). However, this change in $[pH]_i$ was totally abolished when AK-5 cells were treated with peptide inhibitor DEVD (Table 1), and the change was partial when treated with the other peptide inhibitor YVAD, which inhibit apoptosis in these cells. Similarly, transfection of AK-5 cells with Nedd-2 A.S, CrmA or bcl-2 which inhibit the apoptotic process in these cells also abolishes cytoplasmic acidification. These results strongly suggest cytoplasmic acidification as an essential requirement for triggering the apoptotic process. The acidification may be essential for the activity of acid endonuclease (DNase II) which has been implicated in the destruction of the genome [4]. Similarly some of the proteases may require a lower pH for their optimal activity.

Bcl-2 has been shown to protect cells against apoptosis triggered by a variety of stimuli [26] and bcl-2 also inhibits apoptosis in AK-5 cells. In the present study we do not observe any pH change in the bcl-2 transfected clones suggesting again a direct correlation between the cytoplasmic acidification and apoptosis of AK-5 cells (Table 2). Thus our present results suggest acidification as an essential requirement for the cells which are triggered into the apoptotic program and cytoplasmic acidification does not occur when anti-apoptotic gene *bcl-2* and inhibitors of ICE-like proteases are introduced into cells.

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