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Original Paper

Effect of Intracellular Acidity and Ionomycin on Apoptosis in HL-60 Cells

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The aim was to investigate in detail the influence of intracellular pH (pH_i) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) on apoptosis in HL-60 human promyelocytic leukaemia cells. The pH_i was controlled by changing the pH of media as well as by interfering with the pH_i regulatory mechanisms with 3-amino-6-chloro-5-(1-homopiperidyl)-N-(diaminomethylene) pyrazincarboxamide (HMA; an inhibitor of Na^+/H^+ antiport), 4-diisothiocyanatostilbene-2,2′disulfonic acid, (DIDS; an inhibitor of Na^+-dependent HCO_3^-/Cl^- exchange) and nigericin (a K^+ ionophore). The $[Ca^{2+}]_i$ was increased with ionomycin, a Ca^{2+} ionophore. The apoptosis of HL-60 cells was measured with conventional agarose gel electrophoresis for DNA fragmentation and also with the release of ^3H from ^3H-thymidine-labelled DNA. Based on the magnitude of DNA fragmentation and ^3H release at different pH_i, it was shown that apoptosis occurred in HL-60 cells when the pH_i was lowered from normal pH_i of 7.4 to about 7.2–6.7 with a peak increase at pH_i 6.8–6.9. Addition of 4 μM ionomycin to RPMI 1640 medium, which contained 615 μM Ca^{2+}, elevated the apoptosis in the cells. Such an increase in apoptosis by ionomycin in HL-60 cells appeared to result from both an increase in $[Ca^{2+}]_i$ and from a decline in pH_i. The results indicate that the acidic intratumour environment may greatly affect the response of neoplastic tissues to hyperthermia, radiation and chemotherapeutic drugs which cause apoptosis.

Key words: apoptosis, intracellular acidity, ionomycin, HL-60 cells
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INTRODUCTION

IT HAS become increasingly evident that apoptosis plays an important role in embryonic development, controlling immune systems and development of cancer [1–5]. Apoptosis has also been shown to be the major mode of death for cells exposed to radiation, certain chemotherapeutic drugs or hyperthermia [6–13].

The genetic and molecular mechanisms of inducing apoptosis and the cascade of reactions leading to fragmentation of DNA have been intensely investigated in recent years [1–5]. It is generally believed that the internucleosomal fragmentation of DNA to multimers of 180–200 base pairs in apoptosis is carried out by Ca^{2+}/Mg^{2+}-dependent neutral endonuclease(s) including DNase I [2–7, 14–16]. Alternatively, Eastman and colleagues [17–19] reported that the apoptotic degradation of DNA in Chinese hamster ovary cells and HL-60 human promyelocytic leukaemia cells is carried out by Ca^{2+}/Mg^{2+}-independent DNase II, whose optimal pH is near 5.0. These investigators also reported that an increase in intracellular acidity increased apoptosis, and that the elevation of apoptosis by ionomycin, a Ca^{2+} ionophore, resulted not from an increase in the Ca^{2+} concentration, but from an increase in intracellular acidity.

Whatever the nature of the enzyme(s) responsible for the DNA degradation in apoptosis might be, the fact that the acidic environment greatly influences apoptosis of tumour cells may have significant implications in the treatment of neoplastic diseases whose interstitial environment is acidic. Tannock and Rotin [20] and Boyer and Tannock [21] reported that an increase in intracellular acidity by exposing the cells to a low pH environment or by inhibiting the pH_i regulatory mechanisms was cytotoxic and suggested that the pH_i regulatory mechanisms may serve as targets for tumour therapy. We, as well as others, observed that lowering the pH_i also potentiates the hyperthermic cell killing and also enhances

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the response of tumour cells to certain chemotherapeutic drugs [22–27]. The present study was undertaken to reveal the relationship between apoptosis and pH_i, with a view to finding a means of improving the response of tumours to treatment regimens, such as hyperthermia, radiation and certain antineoplastic drugs, which have been reported to cause apoptosis in tumour cells.

MATERIALS AND METHODS

Materials

HL-60 human promyelocytic leukaemia cells were obtained from American Type Culture Collection (Rockville, Maryland, U.S.A.). RPMI-1640 culture medium and fetal bovine serum were obtained from GIBCO/BRL, Life Technologies (Grand Island, New York, U.S.A.). The pH_i probe BCECF-AM (acetyoxymethyl ester of 2′7′-bis-(2-carboxy-ethyl)-5-(and -6) carboxy-fluorescein) and Ca^{2+} probe Fura 2-AM were purchased from Molecular Probes, Inc. (Eugene, Oregon, U.S.A.). Deoxiribonuclease-free ribonuclease and proteinase K were purchased from Boehringer Mannheim (Indianapolis, Indiana, U.S.A.). ^{3}H-labelled thymidine was purchased from Dupont/Nemours & Co (Boston, Massachusetts, U.S.A.). Ionomycin, HMA (3-amino-6-chloro-5-(1-homopiperidyl)-N-(diaminomethylene) pyrazincarboxamide), DIDS (4,4-diiosothiocyanato-stilbene-2,2′-disulphonic acid), nigericin and all other chemicals were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.).

Drug treatment of cells

The HL-60 cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum and 10% penicillin–streptomycin or penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C under humidified 5% CO_2 and 95% air. The mediuim contained 615 μM Ca^{2+} (total Ca^{2+} in RPMI 1640 medium and 20% fetal bovine serum). The cells in exponential growth phase at a cell concentration less than 5×10^5 cells/ml were centrifuged and resuspended at a concentration of 5×10^5 cells/ml in 10 ml of fresh RPMI 1640 medium adjusted to the desired pH with organic buffers: Tris, MOPS and MES (30 mM). The effect of an acidic intracellular environment on apoptosis was studied by treating the cells with the following drugs which lower the pH_i: 10 μM HMA (inhibitor of Na^+/H^+ antiport), 100 μM DIDS (inhibitor of Na^+-dependent HCO_3^-/Cl^- exchange) and 1.0 μg/ml nigericin (K^+ ionophore) alone or in various combinations. The influence of intracellular Ca^{2+} concentration on apoptosis was investigated with the use of 4 μM ionomycin (a Ca^{2+} ionophore). The cells with or without the drugs were incubated in 25 cm^2 plastic culture flasks at 37°C for 4 or 7 h and the DNA degradation was analysed.

Heat-induced apoptosis

The effect of pH on the heat-induced apoptosis was also studied. HL-60 cells were prepared as described above and then suspended in complete RPMI 1640 media in plastic culture flasks; the pH of the media was adjusted to 7.5 or 6.6 by adjusting the CO_2 content in the flasks [22, 24]. The flasks were tightly capped and maintained in a water bath at 37 or 42°C for 1 h [22, 24]. The cells were then incubated in a 37°C incubator for 3 h and subjected to analysis for DNA fragmentation with the use of gel electrophoresis.

DNA gel electrophoresis

The DNA fragmentation resulting from apoptosis was investigated with electrophoresis of DNA extracts. After the various treatments, the cells were washed with phosphate-buffered solution (PBS) and resuspended in lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 10 mM EDTA, proteinase K at 0.1 mg/ml, 1% sodium dodecyl sulphate) and incubated at 48°C for 14 h. Cold 5 M NaCl solution was added to the lysate, and the mixture was vigorously shaken with a vortex and centrifuged at 10 000*g* for 5 min. The supernatant was mixed with isopropylalcohol and stored overnight at −20°C. After centrifugation, the pellet was resuspended in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) and the RNA was digested by adding 0.2 mg/ml DNase-free RNase and incubating at 37°C for 2 h. The DNA content in the final preparation was estimated by spectrophotometry (A260/A280), and 10 μg of DNA from each sample and the DNA molecular weight marker (123 bp ladder, GIBCO/BRL, Life Technologies) were subjected to electrophoresis on a 1.5% agarose gel in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and the DNA was stained with ethidium bromide.

^{3}H-Thymidine release

The degradation of DNA owing to apoptosis was quantitated by measuring ^{3}H release from ^{3}H-thymidine labelled cells. The exponentially growing HL-60 cells, harvested from cultures with fewer than 2×10^5 cells/ml, were incubated with 2.5 μCi/ml of ^{3}H-thymidine for 20 h at 37°C. After washing the cells with medium three times, the cells were treated with various drugs, as described in the previous section, for 4–7 h. The cells were then centrifuged, lysed with lysis buffer (5 mM Tris–HCl pH 7.4, 10 mM EDTA and 0.5% Triton X-100) and centrifuged at 11 000 rpm for 20 min. The ^{3}H radioactivity in the incubation medium (supernatant of first centrifugation after drug treatment), in the supernatant of lysed cells and in the pellet was counted and the per cent ^{3}H release from the labelled DNA was calculated [27].

Determination of intracellular pH (pH_i)

The changes in pH_i of HL-60 cells upon treatment with various drugs, including ionomycin, were measured. The detailed method for the determination of pH_i was previously reported by us and others [22–24]. Briefly, BCECF-AM was dissolved in DMSO at a concentration of 1 μg/μl. The HL-60 cells were suspended in pH 7.5 Tris–MOPS buffered RPMI 1640 medium at approximately 10^7 cells/ml and incubated with 5 μg/ml of BCECF-AM at 37°C for 30 min and washed. The labelled cells were treated with various drugs for 30–240 min, as described before, in microcentrifuge tubes. After the treatment, the cells were centrifuged and resuspended in cuvettes containing Na^+- and HCO_3^--free choline chloride buffer at the same pH used for the drug treatments. The fluorescence intensity of the cells was read with an Hitachi F-2000 fluorometer at excitation wavelengths of 441 and 505 nm, using an emission wavelength of 530 nm. The intracellular pH was obtained from the ratio of fluorescence intensities at 505 and 441 nm excitation, and calibration curves obtained, as described elsewhere [22–25].

Determination of intracellular pH calcium ($[Ca^{2+}]_i$)

To the HL-60 cell suspension in pH 7.5 RPMI 1640 culture media, acetoxymethyl ester of the Ca^{2+} probe Fura-2 (5 μM)

was added and incubated for 30 min at 37°C. The labelled cells were then centrifuged and resuspended at 10^6 cells/ml in 3 ml RPMI 1640 culture media at pH 7.5 or pH 6.6 containing 20% fetal bovine serum. The cells were kept in suspension by magnetic stirring. The $[Ca^{2+}]_i$ was determined by measuring the fluorescence signal of Fura-2 excited at 340 and 380 nm with an emission wavelength of 510 nm in a Hitachi F-2000 fluorescence spectrophotometer equipped with the 2512475-11 option for intracellular cation measurements. The calcium concentration corresponding to a measured 340/380 fluorescence ratio were calculated using the known method of Grynkiewicz and associates [28], in which the maximum and minimum 340/380 ratios are determined at conditions of Ca^{2+} saturation and Ca^{2+} depletion using the methods for membrane perforation by detergent and chelation of Ca^{2+} by EGTA, respectively. When the immediate effect of ionomycin on $[Ca^{2+}]_i$ was investigated, the cells were labelled with the probe first, 9 μg of ionomycin dissolved in ethanol was added to the 3 ml cell suspension in the cuvette using a micropipette to yield an ionomycin concentration of 4 μM and the fluorescence intensity was traced for 10 min. In other studies, the cells were incubated with 4 μM of ionomycin in pH 7.5 or pH 6.6 medium for 4 h at 37°C. The Ca^{2+} probe was added to the cells approximately 30 min before the end of the 4 h incubation. The cells were centrifuged, resuspended in pH 7.5 or pH 6.6 medium and the fluorescence intensity was measured.

RESULTS

Intracellular pH

The changes in pH_i of HL-60 cells upon treatment with various drugs in pH 7.5 and pH 6.6 media are shown in Figure 1. In pH 7.5 medium, the pH_i was 7.40 ± 0.01. The combination of 10 μM of HMA and 100 μM of DIDS (HD, Figure 1) significantly decreased the pH_i of cells in pH 7.5 medium. HMA is an analogue of amiloride, a known inhibitor of Na^+/H^+ antiport and is much more potent and specific than amiloride in increasing intracellular acidity by inhibiting Na^+/H^+ antiport [20–22, 24, 25]. DIDS also increases intracellular acidity by inhibiting HCO_3^-/Cl^- exchange through the cell membrane. Incubation with 4 μM ionomycin (I, Figure 1) in pH 7.5 medium for 4 h slightly reduced the pH_i. It has been shown that ionomycin increases intracellular Ca^{2+} ion concentration and also increases intracellular acidity [18]. Incubation of the cells in pH 7.5 medium with 4 μM of ionomycin together with 10 μM HMA and 100 μM DIDS (IHD, Figure 1) decreased the pH_i to 7.00 ± 0.04, a decline of 0.40 pH units. In pH 7.5 medium, the combination of 1.0 μg/ml nigericin, HMA and DIDS (NHD, Figure 1) decreased the pH_i to 6.95 ± 0.03, which was slightly lower than the pH_i of cells treated with a combination of ionomycin, HMA and DIDS. Nigericin is a K^+ ionophore which lowers the intracellular pH by inducing the influx of H^+ ions in exchange for K^+ ions. When the medium pH was changed from 7.5 to 6.6, the pH_i decreased from 7.40 ± 0.01 to 7.15 ± 0.03 in 4 h. The net declines in pH_i of the cells by the drugs at pH 6.6 were significantly greater than those at pH 7.5. The presence of 4 μM ionomycin in pH 6.6 medium lowered the pH_i to 6.95 ± 0.03, which was 0.20 pH units lower than that of the cells in pH 6.6 medium without drugs. The presence of ionomycin with HMA and DIDS in pH 6.6 medium decreased the pH_i to 6.57 ± 0.02, which was about 0.58 pH units lower than the pH_i of cells in pH 6.6 medium without drugs. Treatment of HL-60 cells in pH 6.6 medium with the combination of nigericin, HMA and DIDS lowered the pH_i to as low as 6.35 ± 0.01 within 30 min.

Intracellular Ca^{2+} concentration

Figure 2 illustrates representative cases which show the effects of ionomycin on $[Ca^{2+}]_i$ in HL-60 cells in pH 7.5 and pH 6.6 media. Upon adding 4 μM ionomycin to the cell suspension, the $[Ca^{2+}]_i$ rapidly rose, peaked at about 1 min and gradually declined. The decline in $[Ca^{2+}]_i$ appeared to result from readjustment of intracellular and extracellular $[Ca^{2+}]_i$ following the initial increase caused by ionomycin. It can be seen that the increase in $[Ca^{2+}]_i$ in pH 6.6 medium by

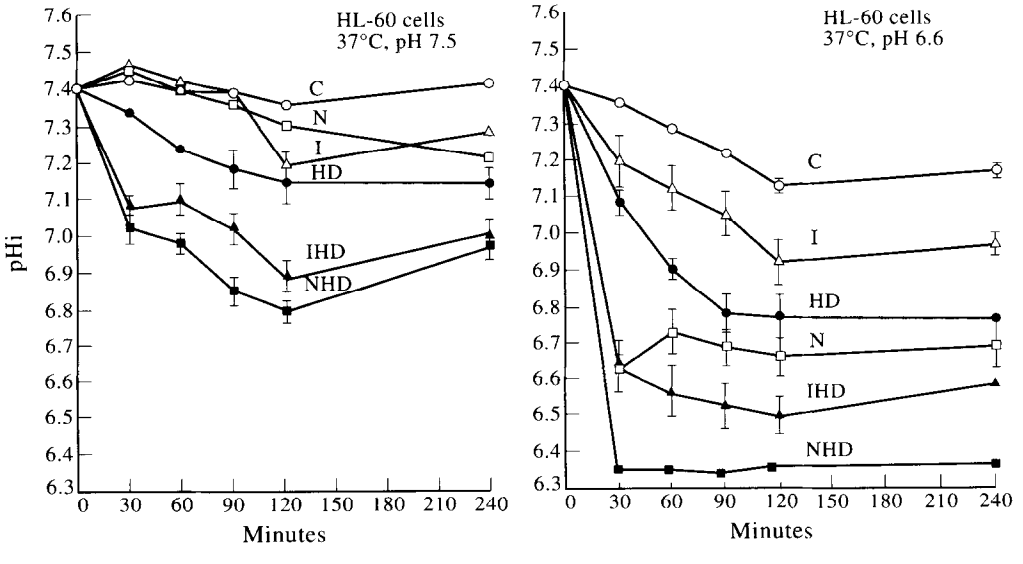

Figure 1. Changes in pH_i of HL-60 cells in the presence of various compounds in pH 7.5 and pH 6.6 media. The cells were maintained in pH 7.5 medium until the experiment was started. Each data point is the mean ± 1 SE for four experiments with quadruplicate samples for each experiment. C, control; N, 1 μg/ml nigercin; I, 4 μM ionomycin; HD, 10 μM HMA plus 100 μM DIDS; IHD, 4 μM ionomycin plus 10 μM HMA plus 100 μM DIDS; NHD, 1 μg/ml nigericin plus 10 μM HMA plus 100 μM DIDS.

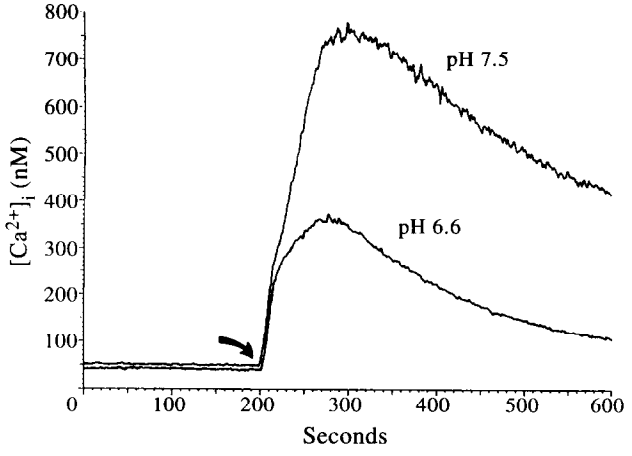

Figure 2. Effects of 4 μM ionomycin on $[Ca^{2+}]_i$ in HL-60 cells at pH 7.5 or pH 6.6. Ionomycin was added at 200 s.

ionomycin was much less than that in pH 7.5 medium. Table 1 shows the $[Ca^{2+}]_i$ measured after incubation of the cells with 4 μM ionomycin for 7 and 240 min. The $[Ca^{2+}]_i$ in control HL-60 cells in pH 7.5 and pH 6.6 media was 65 ± 6 and 54 ± 8 nM, respectively. Upon addition of 4 μM ionomycin, the $[Ca^{2+}]_i$ increased about 7-fold in pH 7.5 medium and 2-fold in pH 6.6 medium in 7 min. The $[Ca^{2+}]_i$ measured after a 4 h incubation with ionomycin was essentially the same as that measured after a 7 min incubation. These results demonstrated that the effect of ionomycin in increasing the Ca^{2+} ion concentration is much greater in neutral pH than in acidic pH.

DNA fragmentation

The fragmentation of DNA in HL-60 cells after incubation in media of different pHs are shown in Figure 3. No noticeable DNA fragmentation occurred in pH 7.5 medium while an incubation of cells for 7 h in media at pH 7.0 occasionally caused a small degree of DNA fragmentation. Faint, but clearly visible DNA ladders were consistently detected in the DNA extract of cells incubated in pH 6.6 medium. The ladder formation was most prominent in pH 6.4 medium, and it disappeared in pH 6.0 medium. The pH_i of HL-60 cells in pH 6.4 and pH 6.0 medium were 6.85 and 6.35, respectively (data not shown), indicating that the DNA fragmentation progressively increases as the pH is decreased to about 6.85 and that DNA fragmentation does not occur at pH_i lower than about 6.35.

The DNA fragmentation in HL-60 cells incubated in pH 7.5 or 6.6 media containing the various drugs are shown in Figure 4. Visible fragmentation of DNA occurred in control HL-60 cells incubated without drugs in pH 6.6 medium, but not in pH 7.5 medium. The presence of 10 μM HMA plus 100 μM DIDS (HD) caused no DNA fragmentation in

Table 1. *Effect of ionomycin* (4 μM) *on* $[Ca^{2+}]_i$ *in HL-60 cells*

	Control	7 min	4 h
pH 7.5	65 ± 6	440 ± 108	436 ± 21
pH 6.6	54 ± 8	98 ± 14	127 ± 5

Averages of between six and seven measurements ± SE are shown.

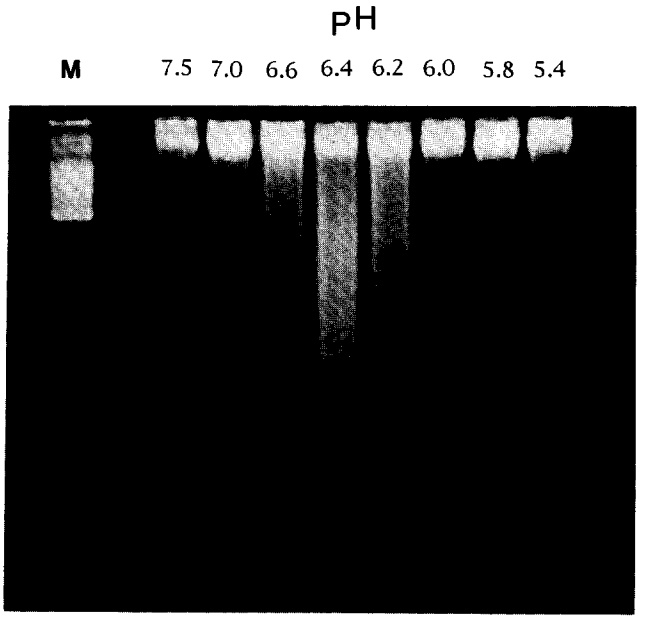

Figure 3. Agarose gel electrophoresis of DNA from HL-60 cells incubated for 7 h in media of different pHs. Note that the pH is the pH of media, and thus they are different from pH_i.

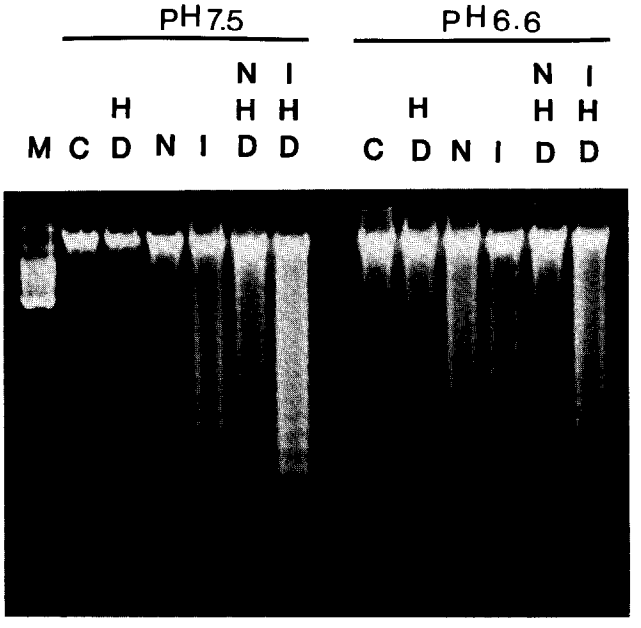

Figure 4. Agarose gel electrophoresis of DNA from HL-60 cells incubated for 7 h in pH 7.5 or 6.6 medium containing various drugs: M, DNA molecular weight marker; C, control; HD, 10 μM HMA plus 100 μM DIDS; N, 1 μg/ml nigericin; I, 4 μM ionomycin; NHD, 1 μg/ml nigericin plus 10 μM HMA plus 100 μM DIDS; IHD, 4 μM ionomycin plus 10 μM HMA plus 100 μM DIDS. Note that the pH 7.5 or 6.6 refer to the pH of media, and thus they are different from pH_i.

pH 7.5 medium while it caused visible DNA fragmentation in pH 6.6 medium, indicating these drugs are non-toxic to the cells in a neutral pH environment at the concentrations used in the present study. Incubation of HL-60 cells with 1.0 μg/ml nigericin (N), which lowers pH_i by causing an efflux of K^+ ions from cells in exchange for an influx of H^+ ions (Figure 1),

induced slight DNA fragmentation in pH 7.5 medium and marked DNA fragmentation in pH 6.6 medium. The combination of nigericin, HMA and DIDS (NHD) caused significant DNA fragmentation in the cells in pH 7.5 while the combination of the three drugs failed to cause DNA fragmentation in the cells in pH 6.6 medium. In pH 7.5 medium, marked DNA fragmentation was caused by 4 μM of ionomycin (I) and the DNA fragmentation was increased further in the presence of a combination of ionmycin, HMA and DIDS (IHD). The DNA fragmentation by ionomycin alone (I) or with HMA and DIDS (IHD) in pH 6.6 medium were far less than those in pH 7.5 medium.

^{3}H release

Figure 5 shows that 14–20% of ^{3}H was released from the control cells after an incubation for 4–7 h in both pH 7.5 or 6.6 media. The ^{3}H release after incubation with a combination of 10 μM HMA and 100 μM DIDS (HD) in pH 6.6 medium or 7.5 medium was only slightly larger than that in the corresponding control. Incubation of ^{3}H-labelled cells with 4 μM of ionomycin (I) for 4 h resulted in a 74.2 ± 6.1% ^{3}H release in pH 7.5 medium in contrast to only a 42.2 ± 4.1% ^{3}H release in pH 6.6 medium. When the cells were incubated for 4 h with 4 μM ionomycin in combination with 10 μM HMA and 100 μM DIDS (IHD), 92.1 ± 4.7% of the ^{3}H was released in pH 7.5 medium while only 49.9 ± 9.1% of the ^{3}H was released in pH 6.6 medium. Incubation of cells for 7 h with 1.0 μg/ml nigericin (N) in pH 6.6 medium resulted in a 54.0 ± 7.6% ^{3}H release while that in pH 7.5 medium resulted in a 29.0 ± 6.4% ^{3}H release. The ^{3}H release after a 7 h incubation with the combination of 1 μg/ml nigericin, 10 μM HMA and 100 μM DIDS (NHD) in pH 6.6 medium was only 9.6 ± 1.8%, while that in pH 7.5 medium was 64.7 ± 10.3%. These results were in good agreement with the DNA fragmentation results shown in Figure 4.

Effects of heating on DNA fragmentation

Figure 6 shows that heating at 42°C for 1 h induced a noticeable degree of DNA fragmentation in HL-60 cells in pH 7.5 medium demonstrating that hyperthermia caused

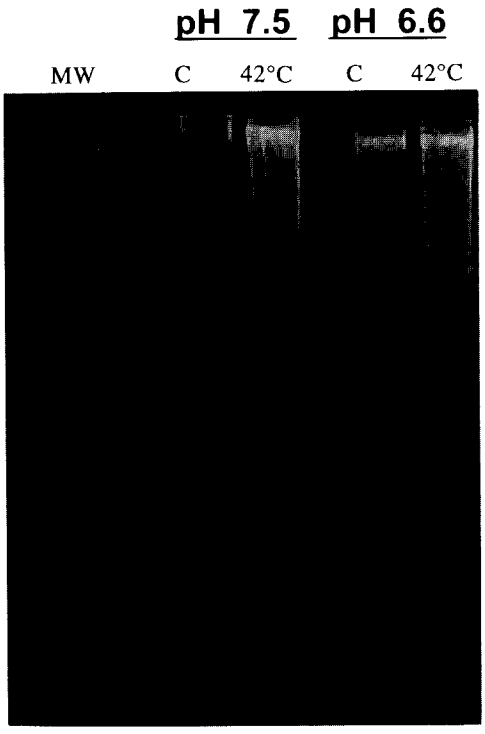

Figure 6. Agarose gel electrophoresis of DNA from control HL-60 cells and that from HL-60 cells heated at 42°C for 1 h in pH 7.5 or 6.6 media. After heating, the cells were incubated at 37°C for 3 h and subjected to gel electrophoresis for DNA fragmentation.

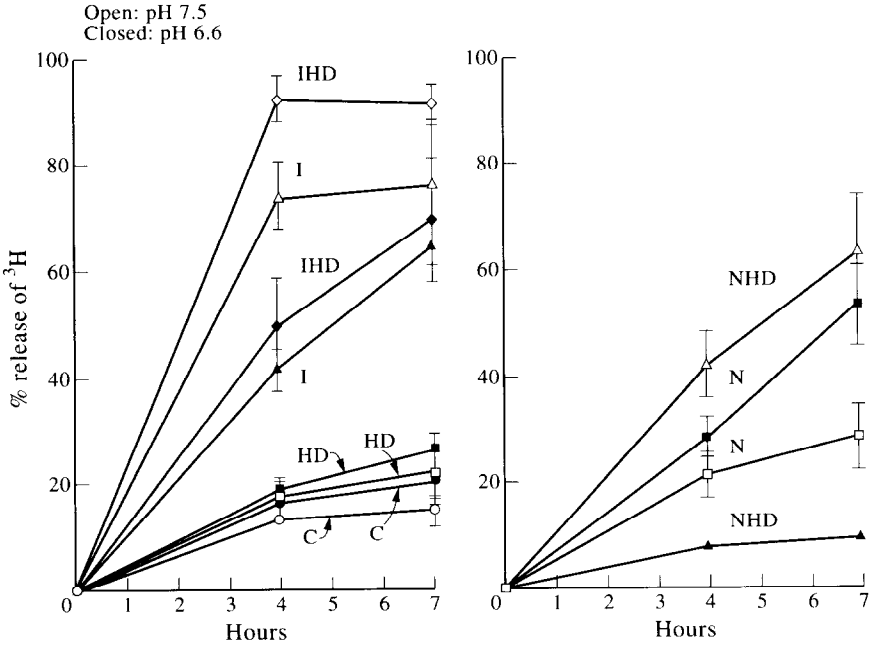

Figure 5. ^{3}H release from HL-60 cells labelled with ^{3}H-thymidine upon incubation in pH 7.5 or pH 6.6 containing various drugs: C, control; HD, 10 μM HMA plus 100 μM DIDS; N, 1 μg/ml nigericin; I, 4 μM ionomycin; NHD, 1 μg/ml nigericin plus 10 μM HMA plus 100 μM DIDS; IHD, 4 μM ionomycin plus 10 μM HMA plus 100 μM DIDS. The open symbols are the ^{3}H release at pH 7.5 and the closed symbols are the ^{3}H release at pH 6.6.

apoptosis. Lowering the medium pH to 6.6 enhanced the heat-induced DNA fragmentation.

DISCUSSION

It is demonstrated in the present study that both intracellular acidity and intracellular Ca^{2+} concentration play important roles in the apoptosis of HL-60 cells. As shown in Figure 3, the DNA fragmentation started to occur as the medium pH was lowered to below 7.0, became significant at 6.6 and peaked at 6.4. Note that the pH_i of HL-60 cells in pH 6.6 and pH 6.4 medium was 7.15 and 6.85, respectively, indicating that the DNA of HIL-60 cells degrades when the pH_i is lowered to 7.15–6.85. The DNA fragmentation (Figure 4) and ^3H release (Figure 5) demonstrated that incubation of HL-60 cells without drugs in pH 6.6 medium caused noticeable DNA degradation, not seen at pH 7.5. Furthermore, treating the cells with nigericin (N) or HMA plus DIDS (HD) induced more DNA degradation in pH 6.6 medium than in pH 7.5 medium. Note that nigericin or HMA with DIDS lowered the pH_i of HL-60 cells to 7.2–7.3 in pH 7.5 medium and to 6.7–6.8 in pH 6.6 medium (Figure 1). It could then be concluded that the greater DNA degradation in pH 6.6 medium than in pH 7.5 medium either with or without the drugs was a result of the greater decline in pH_i of the cells in pH 6.6 medium than in pH 7.5 medium.

The treatment of HL-60 cells in pH 7.5 medium with the combination of nigericin, HMA and DIDS (NHD) lowered the pHi to 7.0 and caused significant DNA degradation. Interestingly, the same drug combination in pH 6.6 medium failed to induce DNA degradation (Figures 4 and 5). This lack of DNA degradation in pH 6.6 medium by the three-drug combination, i.e. NHD, could be attributed to the decline in pH_i to as low as 6.4, as shown in Figure 1. This conclusion is consistent with the results shown in Figure 2 that no DNA fragmentation occurred in the cells incubated in pH 6.0 medium, at which the pH_i was about 6.4 (data not shown). Overall, one may conclude that apoptotic cell death takes place in HL-60 cells when the pH_i of the cells is lowered to 6.7–7.2 with a peak DNA degradation occurring probably in the pH_i 6.8–6.9 range.

Ionomycin at 4 μM caused marked DNA degradation and the combination of ionomycin with HMA and DIDS (IHD) was more potent than ionomycin alone in inducing DNA degradation as indicated by the DNA fragmentation (Figure 4) and ^3H release (Figure 5). DNA degradation by ionomycin alone or in combination with HMA and DIDS in pH 6.6 medium was less than that in pH 7.5 medium. The smaller increase in $[Ca^{2+}]_i$ by ionomycin in pH 6.6 medium than in pH 7.5 medium, as illustrated in Figure 2 and Table 1, may account for this pH dependent difference in the potency of ionomycin to increase DNA degradation. Barry and associates [17–19] suggested that the increase in DNA fragmentation by ionomycin results from an increase in intracellular acidity and not from an increase in $[Ca^{2+}]_i$. As shown in Figure 1, ionomycin indeed caused a decline in pH_i in the present study. For example, the pH_i of cells treated with the combination of ionomycin, HMA and DIDS was notably lower than the pH_i of cells treated with the combination of HMA and DIDS in both pH 7.5 and pH 6.6 medium. The greater DNA degradation in the presence of ionomycin, HMA and DIDS relative to that in the presence of HMA and DIDS (Figures 4 and 5) may then appear to be caused by the decline in pH_i due to the ionomycin. However, we conclude that the increase in DNA degradation by ionomycin in HL-60 cells results, at least in part, from an increase in the intracellular Ca^{2+} concentration based on the following facts. First, as alluded to above and shown in Figure 2 and Table 1, the increase in $[Ca^{2+}]_i$ by ionomycin in pH 6.6 medium was significantly less than that in pH 7.5 medium. The fact that DNA degradation in pH 6.6 medium containing ionomycin alone or other drugs were less than that in pH 7.5 medium containing the same drugs, despite the fact that the pH_i of the cells in pH 6.6 medium was lower than that in pH 7.5 medium, unequivocally demonstrate the importance of $[Ca^{2+}]_i$ in apoptosis in HL-60 cells. Second, as shown in Figure 1, the pH_i of the cells in the presence of 4 μM of ionomycin was higher than that in the presence of HMA plus DIDS in both pH 7.5 and pH 6.6 medium. If the reduction of pH_i is the only mechanism by which ionomycin induces DNA degradation, then ionomycin would be less potent than HMA plus DIDS in inducing DNA degradation. However, the results in Figures 4 and 5 show that the DNA degradations caused by ionomycin were far greater than those caused by HMA plus DIDS. Third, in both pH 7.5 and 6.6 medium, the DNA degradation caused by ionomycin in combination with HMA and DIDS (IHD) was far greater than those caused by nigericin in combination with HMA and DIDS (NHD), while the pH_i of the cells treated with the combination of ionomycin, HMA and DIDS was higher than that of the cells treated with the combination of nigericin, HMA and DIDS. This fact again suggests that an increase in $[Ca^{2+}]_i$ by ionomycin plays an important role in the DNA degradation caused by ionomycin alone or in combination with other drugs.

HMA, DIDS and nigericin alone did not cause noticeable DNA degradation in the pH 7.5 environment (Figures 4 and 5) demonstrating that they are non-toxic at the concentrations we used. Tannock and his colleagues have previously observed that these drugs were non-toxic and did not affect the clonogenicity of tumour cells in a neutral pH environment [20–25]. However, the results of the present studies demonstrate that these drugs do lower the pH_i and kill the cells through apoptosis in an acidic environment. Note that the pH_i is lowered even in a neutral pH environment by these drugs, but the decline is not extensive enough to be toxic to the cells.

It has been known that hyperthermia causes apoptosis [3]. The results shown in Figure 6 clearly demonstrated that acidification of the intracellular environment enhances the heat-induced apoptosis. In our previous studies, we observed that an administration of HMA, DIDS and nigericin to mice bearing tumours enhanced the response of tumours to hyperthermia [23, 24]. It is highly likely that the decrease in pH_i by the drugs increased the heat-induced apoptosis in the tumours *in vivo*. It is known that heating lowers the pH_i and also lowers the intratumour pH [22–25, 29]. We previously reported that a significantly greater number of cells died in tumours left *in situ* after heating than in tumours removed immediately after heating [30]. Such additional cell death after heating in tumours may be attributed, at least in part, to apoptotic cell death as a result of the heat-induced acidification of the intratumour environment. It is also known that an acidic environment increases the cytocidal effects of many antineoplastic drugs [27]. The relationship between the effect of the antineoplastic drugs, environmental acidity and apoptosis remains to be elucidated.

In summary, apoptotic fragmentation of DNA in HL-60 cells increased as the pH_i was lowered to the 6.7–7.2 range

with a peak DNA fragmentation occurring at pH_i 6.8–6.9. The mechanisms by which ionomycin increased the apoptosis of HL-60 cells is not clear, but both an increase in $[Ca^{2+}]_i$ and a decline in pH_i appear to be involved. The close relationship between the intracellular pH and apoptosis may have significant implications in the tumour response to various therapies, such as radiation, hyperthermia and some chemotherapeutic drugs, which cause apoptosis in tumour cells.

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