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Original Article

Effects of Intracellular pH on Apoptosis in HL-60 Human Leukemia Cells

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The nature of the endonucleases responsible for DNA fragmentation in apoptosis has not yet been clearly defined. The intracellular acidity has been known to greatly affect apoptosis probably by affecting the activity of the endonucleases. In this study, the implication of pH in the apoptosis was investigated through the use of human HL-60 leukemia cells. The cells were incubated in medias with different pH ranging from 3.5 to 7.5 for 4 hrs and the mode of cell death was investigated. The trypan blue exclusion assay showed that close to 25% and 90% of the cells were dead when incubated in pH 6.4 and pH 5.0 media, respectively. The agarose gel electrophoresis of DNA demonstrated that significant DNA fragmentation occurred in the HL-60 cells incubated in the pH 6.2-6.4 media for 4 hr indicating cell death by apoptosis. The electron microscopy study also demonstrated that many of the cells incubated in the pH 6.4 medium were in the process of apoptosis while the cells maintained in the pH 5.0 medium were dying by necrosis. The intracellular pH (pHi) of HL-60 cells was 6.6-6.9 when the extracellular pH (pHe) was 6.2-6.4. These results demonstrated that DNase I which has a maximal endonuclease activity near pH 7.0 may be responsible for the apoptosis accompanied by DNA fragmentation in HL-60 cells in the pH 6.4 medium. This observation is at variance with the previous reports that DNase II mediate the DNA fragmentation in apoptosis. The cell death at extremely low pH (pH 5.0) appeared to be due mainly to necrosis.

Key Words: Apoptosis, intracellular acidity, HL-60 cells

Apoptosis is a form of cell death which takes place following a programmed, active and gene-controlled molecular and biochemical process (Cohen, 1993; Wyllie, 1993; Green *et al.* 1994; Stewart, 1994). Apoptosis is an essential process in morphogenesis, hormonal regulation, immune response, and oncogenesis. In addition, apoptosis has been demonstrated to be the major mode of cell death in response to damage caused by external insults such as chemotherapeutic drugs, ionizing radiation, and hyperthermia (Kaufmann, 1989; Barry *et al.* 1990; Harmon *et al.* 1990; Baxter and Lavin, 1992; Langley *et al.* 1993; Meyn *et al.* 1993; Waddick *et al.* 1993). Apoptosis differs from necrotic cell death, the other mode of cell death, which occurs in an uncontrolled manner when cells are extensively injured.

Several pro-apoptotic genes such as *fas*, *c-myc*, and p53 genes and anti-apoptotic genes such as *bcl-2* gene have been implicated in cell death due to various causes (Korsmeyer, 1992; Chen *et al.* 1994; Miyashita *et al.* 1994; Brunner *et al.* 1995). The morphological change of cells in apoptosis are rather different from that of necrotic cells. One of the biochemical characteristics of apoptosis is the fragmentation of internucleosomal DNA into multimers

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of 180-base pair by endonuclease(s) (Nikonova *et al.* 1982; Arends *et al.* 1990; Martin and Cotter, 1994; Stewart, 1994). However, in certain instances, cells may die through apoptosis with DNA fragmentation not of 180 bp but of much larger fragments (Brown *et al.* 1993). In such cases, a "ladder" is not observed in the agarose gel electrophoresis of DNA, which is often referred to as the hallmark of apoptosis.

A number of investigators have demonstrated that Ca^{++}/Mg^{++} dependent DNase I, which is maximally active at pH near 7.0 is responsible for the DNA fragmentation in apoptosis (Nikonova *et al.* 1982; Arends *et al.* 1990; Meyn *et al.* 1993; Waddick *et al.* 1993; Wyllie, 1993; Martin and Cotter, 1994; Stewart, 1994). On the other hand, other investigators (Barry *et al.* 1990; Barry and Eastman, 1992; Barry *et al.* 1993; Barry and Eastman, 1993) asserted that apoptosis is caused not by DNase I but by Ca^{++}/Mg^{++} independent and acidic DNase II. In support of their contention, they pointed out that the DNA of HL-60 cells and chinese hamster cells (CHO cells) fragmented when the cells were incubated in extremely acidic media of pH below 5.0. In direct contrast to this contention that the acidification of the intracellular environment is essential for apoptosis, Furuya *et al.* (1994) reported that there was no acidification in the intracellular pH during apoptosis of prostatic cancer cells caused by thapsigargin. In the present study, the relationship between pH and the apoptosis in HL-60 cells was investigated to gain further insight into the mechanism of biochemical process responsible for the DNA fragmentation in apoptosis. Specifically, the effect of different pHi on the mode of cell death, i.e. apoptosis and necrosis, was assessed to reveal the major endonucleases responsible for apoptosis.

MATERIAL AND METHODS

Cells

Human promyelocytic leukemic HL-60 cells were obtained from American Types Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 ug/ml) under 5% CO_2 and 95% air atmosphere at 37℃. The cell concentration was maintained bellow 5×10^5 cells/ml and all the experiments were done with the cells in the exponential growth phase.

Determination of pHi

The effects of extracellular pH (pHe) on the intracellular pH (pHi) in HL-60 cells was determined. The pHi was determined with the use of pH probe BCECF-AM (acetyoxymethyl ester of 2'7'-bis-(2-carboxy-ethyl)-5-(and-6)carboxyl-fluorescein) (Kim *et al.* 1991; Song *et al.* 1994 a, 1994b). The cells were labeled with BCECF-AM by incubating the cells with 5 ug/ml of BCECF-AM in pH 7.5 RPMI medium at 37℃ for 30 min. The cells loaded with the pH-probe were suspended in mediums of different pH in microcentrifuge tubes and were incubated for 1 hr at 37℃. The cells were centrifuged and resuspended in cuvettes containing Na^+ and HCO_3^- free chloride buffer at the same pH used for the incubation and the fluorescence intensity was read immediately. The pHi was estimated from the fluorescence intensity and the calibration curve obtained as previously described (Song *et al.* 1994 a, 1994b).

Incubation of cells in different pH media

The pH of RPMI 1640 medium was adjusted to 3.5-7.5 using Tris, MOPS (30 mM), and MES (30 mM) buffer. HL-60 cells in the exponential growth phase were centrifuged and suspended in 10 ml of the fresh RPMI media of different pH at $4\text{-}5\times10^5$ cells/ml. The cells were maintained in a 5% CO_2 and 95% air incubator at 37℃ for 4 hrs and the viability of the cells, DNA fragmentation, and morphological changes were investigated.

Gel electrophoresis for DNA

The cells were washed with PBS and suspended 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 sulfate) and incubated for 14 hrs at 48℃. After vigorously

at pHe 6.4 were stained. More than 90% of cells maintained at pHe 5.0 were stained by trypan blue demonstrating that a 4 hr exposure of cells to pH 5.0 environment killed almost all the cells.

The morphologic changes observed with electron microscopy were also in agreement with the aforementioned results as shown in Fig. 4. The morphological feature of control HL-60 cells maintained at pHe 7.5 is shown in Fig. 4a. The electron micrograph of HL-60 cells maintained at pHe 6.4 for 4 hrs shows extensive nuclear and cell surface protrusions (Fig. 4b). Some of the apoptotic bodies with varying size and composition including condensed chromatin are still membrane-bound while others are separated from the main body. Fig. 4c is the electron micrograph of HL-60 cells maintained at pH 5.0 for 4 hrs showing apparent necrosis. The chromatin and most of the cytoplasmic components are degraded and the plasma membrane was ruptured.

DISCUSSION

The results obtained in the present study unequivocally demonstrated that HL-60 cells undergo apoptotic death when they are maintained in pH 6.2-6.4 media while they die mainly through necrosis upon exposure to extremely acidic media (pH 5.0).

As alluded to previously, a number of investigators reported that apoptosis accompanied by DNA fragmentation is caused by Ca^{++}/Mg^{++} dependent DNase I whose optimal pH is about 7.0 (Nikonova *et al.* 1982; Arends *et al.* 1990; Meyn *et al.* 1993; Waddick *et al.* 1993; Wyllie, 1993; Martin and Cotter, 1994; Stewart, 1994). As can be seen in Fig. 2, significant DNA fragmentation occurred in HL-60 cells indicating apoptosis occurred when the cells was incubated for 4 hr in pH 6.2-6.4 media. The electron microscopy study also demonstrated that some of HL-60 cells were in the process of apoptosis at pHe 6.2-6.4 (Fig. 4). Note that the pHi of HL-60 cells in pH 6.2-6.4 media was 6.6-7.0 (Fig. 1), indicating that HL-60 cells, like other mammalian cells, possess powerful pHi regulatory mechanisms. It has been known that the pHi regulatory mechanisms present drastic change in pHi when cells are exposed to non-physiological pH environment or to unusual amount of acidic metabolites (Haveman, 1979; Roos and Boron, 1981; Song *et al.* 1993; Tannock and Rotin, 1993). The fact that the apoptosis and DNA fragmentation occured at pHe 6.2-6.4, i.e. pHi 6.6-7.0, in HL-60 cells in the present study strongly indicated that apoptosis is caused by DNase I.

The trypan blue exclusion assay (Fig. 3) demonstrated that about 25% of the HL-60 cells died at pHe 6.4 and practically all the HL-60 cells exposed to pH 5.0 medium for 4 hr died. Note that the dye exclusion assay demonstrates only the viability of the cells and it does not indicate whether the cells die by apoptosis or necrosis. It has been known, however, that the cells in apoptosis usually exclude dye uptake (Darzynkiewicz et al. 1994) and thus it is highly likely that the cells which showed trypan blue uptake at pHe 6.4 were dead by necrosis. Since no "ladder" was found in the gel electrophoresis of the DNA from the cells exposed to the media with pH 5.0 or lower, it could be concluded that cell death as a consequence of an exposure to the acidic media was through necrosis. The result of electron micrograph support this conclusion (Fig. 4c). It should be pointed out, however, that not all apoptotic cell death results in DNA fragmentation detectable with gel electrophoresis. The breaking of DNA to larger than 50 kilobase pair frequency have been reported to be sufficient to induce morphological apoptosis although the familiar "ladder" may not be demonstrated in gel electrophoresis of DNA (Brown *et al.* 1993). Therefore, the possibility that some of the cells death at the extremely low pH environment in the present study was due to apoptosis may not be completely excluded.

The observations and conclusions made in the present study that apoptosis occurs when the intracellular pH is near 7.0 and that DNase I, which is maximally active at pH 7.0, is responsible for the DNA fragmentation in apoptosis in HL-60 cells is in general agree-

ment with the report by Furuya *et al.* (1994) that pHi in prostatic cancer cells was near 7.0 during apoptosis. My results were at variance with a sereis of reports from Eastman's laboratory (Barry *et al.* 1990; Barry and Eastman, 1992; Barry *et al.* 1993; Barry and Eastman, 1993). These investigators concluded that apoptosis in HL-60 cells as well as in other cells was due to an activation of acidic DNase II and that apoptosis occurred when the intracellular pH was lowered below 5.0. The cause of the discrepancy between the results in the present study and the observations reported by Eastman and his associates is unclear. I have observed that when HL-60 cells were exposed to an extremely acidic media (pH 3.0-5.0) for a prolonged period the DNA degraded and produced a smear in the agarose gel electrophoresis of the DNA. The cells were apparently necrotic as shown in Fig 4. It is possible that the aforementioned investigations interpreted the formations of the smear, which is a product of random degradation of DNA, as a sign of apoptosis. It is of interest that Li and Eastman(1995) recently reported that acidic pHi did not appear essential for apoptosis, which is in direct contrast to their previous contention. This conclusion may be interpreted to suggest that acidic DNase II is not necessary involved in apoptosis. Work is in progress to further characterize the endonuclease responsible for apoptosis using different cell lines.

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