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Cell cycle arrest and apoptosis of leukemia cells induced by L-asparaginase

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Apoptotic cell death of murine leukemia cells induced by *E. coli* L-asparaginase was studied. Deprivation of L-asparagine from the culture of L5178Y cells by L-asparaginase caused the fragmentation of chromosomal DNA of the leukemia cells within 24 h. Prior to the degradation of DNA, cell cycles of L5178Y cells were found to be arrested in G1 phase, and evidence of the DNA strand breaks was initially observed in G1 phase cells as early as 8 h after the asparaginase treatment. Therefore, apoptosis of leukemia cells induced by L-asparaginase is an event that is associated with the cell cycle arrest in G1 phase.
Keywords: L-asparaginase; apoptosis; cell cycle; G1 arrest; leukemia

Introduction

L-Asparaginase (EC 3.5.1.1), which catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia, has been used as an effective therapeutic agent for the treatment of acute lymphoblastic leukemia and lymphosarcoma.[1,2] Horowitz *et al*[3] found that leukemia cells that are killed by L-asparaginase have low levels of asparagine synthetase activity. It was suggested that these cells depend on an external supply of L-asparagine, and that when L-asparagine is hydrolyzed by L-asparaginase, the cells die. However, no further studies have been conducted to clarify the biochemical events leading to the death of leukemia cells treated with L-asparaginase. Recently, Story *et al*[4] reported that murine and canine lymphoma cells treated with L-asparaginase undergo apoptosis, as evidenced by characteristic changes of cell morphology and internucleosomal DNA fragmentation.[5–7] Apoptotic cell death is known to be intimately linked to the cell cycle.[8] Therefore, in the present study, we analyzed the cell cycle distribution and apoptosis of murine leukemia cells treated with L-asparaginase.

Materials and methods

Materials

L-Asparaginase from *E. coli* was kindly provided by Kyowa Hakko Kogyo (Tokyo, Japan). Murine leukemia cells (L5178Y) and murine melanoma cells (B16) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the Seikagaku Corporation (Tokyo, Japan), respectively. Propidium iodide, thymidine, deoxycytidine and colcemid were obtained from Sigma (St Louis, MO, USA). Other reagents were of analytical grade.

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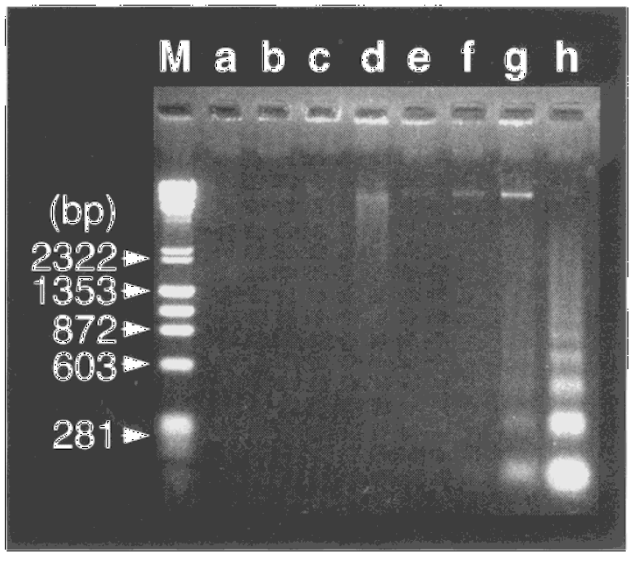

Figure 1 Agarose gel electrophoresis of DNA extracted from L5178Y cells and B16 cells treated with L-asparaginase (100 IU/ml). Lanes a, b, c and d: B16 cells treated with L-asparaginase for 0, 8, 16 and 24 h, respectively. Lanes e, f, g and h: L5178Y cells treated with L-asparaginase for 0, 8, 16 and 24 h, respectively. Lane M: Molecular weight markers, *Hin*dIII-digested bacteriophage λ DNA and *Hae*III-digested φX174 DNA.

Cell culture and asparaginase treatment

Both L5178Y cells and B16 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C under a humidified 5% CO_2 atmosphere. L5178Y cells and B16 cells (5×10^5 cells/ml) were incubated in the presence of L-asparaginase (100 IU/ml) for 0, 8, 16 and 24 h.

L5178Y cells synchronized in M phase were prepared as described by Doida *et al*.[9] Briefly, excess thymidine (2.5×10^{-3}M) was added to exponentially growing L5178Y cells (5×10^5 cells/ml) to inhibit *de novo* synthesis of deoxycytidine for 5 h. These cells, most of which were in the S phase, were then allowed to proceed with the cell cycle and to be arrested in M phase. This was accomplished by incubating the cells in the absence of thymidine and the presence of deoxycytidine (10^{-4}M) and colcemid (0.025 μg/ml) for another 5 h.

DNA-fragmentation analysis

Oligonucleosome-sized DNA was extracted as described[10] and was analyzed by gel electrophoresis in 1.8% (w/v) agarose gel containing 0.01% ethidium bromide. The DNA fragments were visualized by illumination of UV light.

Flow cytometry analysis

Flow cytometry analysis was conducted on L5178Y cells fixed and permeabilized with formaldehyde and ethanol. For cell cycle analysis, nuclear DNA was stained with propidium iodide.[11] For detection of DNA strand breaks in individual apoptotic L5178Y cells, the 3′-hydroxyl termini of DNA breaks were labeled with biotin-dUTP and terminal deoxynucleotidyl transferase (TdT), and were subsequently stained with fluoresceined avidin.[12] Two-color flow cytometry analysis for DNA strand breaks and cell cycle was conducted using FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA, USA).

Results and discussion

DNA strand breaks in L5178Y cells treated with L-asparaginase

Figure 1 shows the time course of the fragmentation of chromosomal DNA of L5178Y leukemia and B16 melanoma cells treated with L-asparaginase. A sample of DNA extracted from L5178Y cells treated with L-asparaginase for 16 and 24 h showed DNA ladders with sizes equivalent to single and multiple nucleosomes (Figure 1g and h). In contrast, DNA fragmentation was not observed in B16 melanoma cells treated with L-asparaginase (Figure 1a–d).

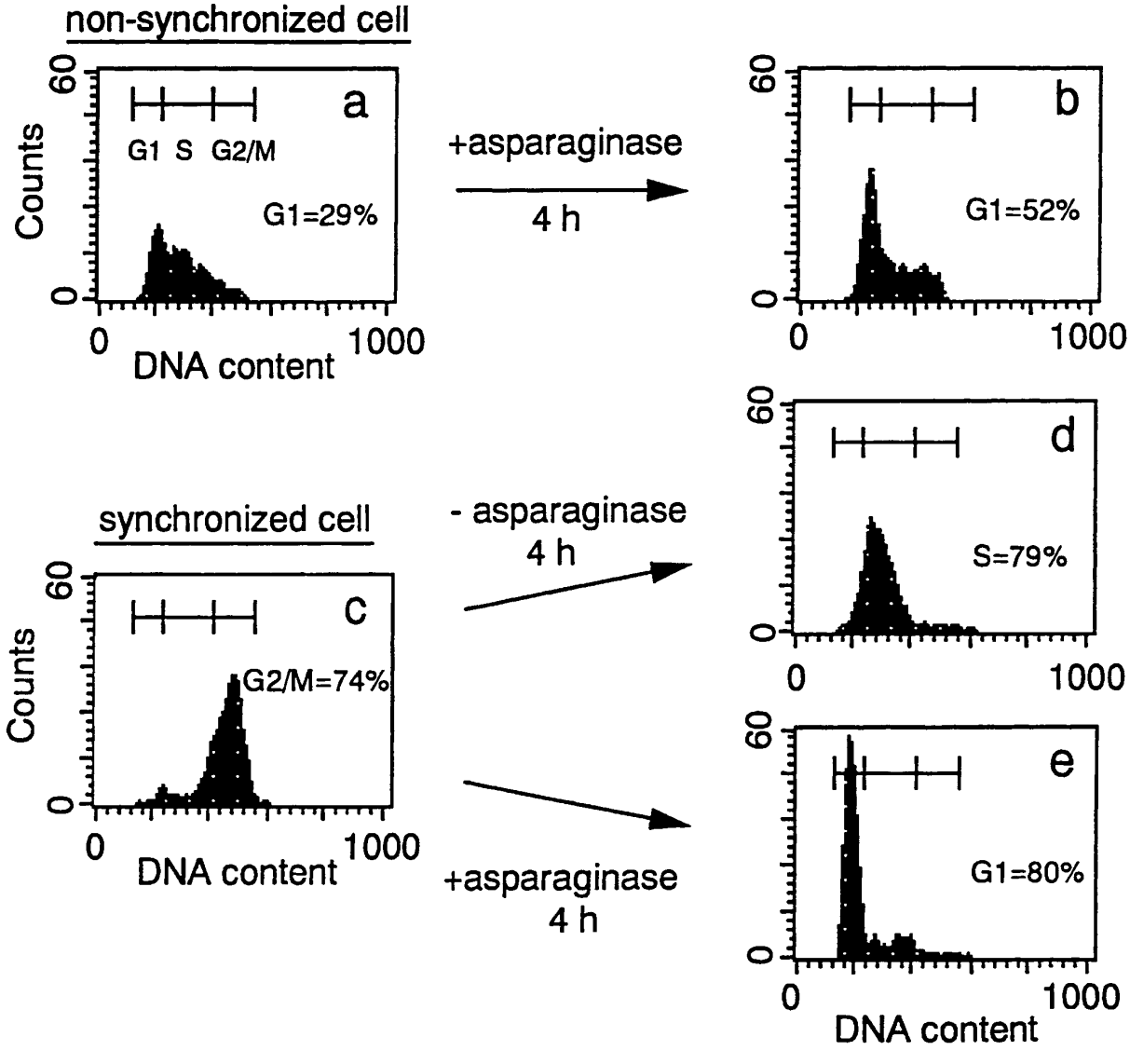

Figure 2 Cell cycle change of L5178Y cells treated with L-asparaginase. (a and b) normal L5178Y cells before and after the treatment with L-asparaginase for 4 h, respectively. (c and e) synchronized L5178Y cells before and after the treatment with L-asparaginase for 4 h, respectively. (d) synchronized L5178Y cells incubated in the absence of L-asparaginase for 4 h. The cell cycle distributions were: (a) 29% (G1), 60% (S), 11% (G2/M); (b) 52% (G1), 42% (S), 6% (G2/M); (c) 4% (G1), 22% (S), 74% (G2/M); (d) 16% (G1), 79% (S), 3% (G2/M); (e) 80% (G1), 17% (S), 3% (G2/M).

Cell cycle arrest induced by L-asparaginase

We then sought to observe whether the cell cycle distribution in L5178Y cells is altered by treatment with L-asparaginase. Figure 2a shows the cell cycle distribution of L5178Y cells exponentially growing in the normal medium in the absence of L-asparaginase; 29% in G1 phase, 60% in S phase and 11% in G2/M phase. Figure 2b shows the profile of L5178Y cells treated with L-asparaginase (100 IU/ml). There was a significant increase in the proportion of cells in G1 phase (52%) as early as 4 h after the addition of L-asparaginase. To clarify whether the increase of G1 cells was due to the cell cycle arrest in G1 phase, L5178Y cells synchronized in M phase were cultured either in the presence or absence of L-asparaginase. Figure 2c shows the cell cycle distribution of L5178Y cells synchronized in M phase; 4% in G1 phase, 22% in S phase and 74% in G2/M phase. These cells were then allowed to proceed with the cell cycle either in the absence or the presence of L-asparaginase for 4 h (Figures 2d and e). Since one round of cell cycling in L5178Y cells consists of G1 phase (1.5 h), S phase (5–6 h) and G2/M phase (1.6 h),[13] the cells were expected to be in S phase after a 4 h culture in the absence of L-asparaginase. As expected, most of the synchronous cells were found to be in S phase; 16% in G1 phase, 79% in S phase and 5% in G2/M phase (Figure 2d). On the other hand, in the presence of L-asparaginase, most of the cells were found to be in G1 phase after the same incubation time; 80% in G1 phase, 17% in S phase and 3% in G2/M phase (Figure 2e). Therefore, treatment of L5178Y cells with L-asparaginase resulted in the cell cycle arrest in G1 phase.

DNA strand breaks and cell cycle phases

To see whether apoptosis takes place in cells arrested in G1 phase, cells treated with L-asparaginase were fixed with formaldehyde and ethanol, and subsequently subjected to the simultaneous analysis of DNA strand breaks and cell cycles. DNA strand break was not detected in exponentially growing L5178Y cells (Figure 3a). As early as 8 h after the treatment with L-asparaginase, small numbers of G1 cells with DNA strand breaks were observed, as shown in the R2-region of Figure 3b. Higher numbers of apoptotic cells with DNA strand breaks were observed by increasing the incubation time (Figures 3c and d). Most of these cells were in G1 phase. These data suggested that apoptosis was induced in leukemia cells that were arrested in G1 phase.

Apoptosis in late G1 phase is commonly observed in cells responding to death signals induced by DNA damage or deprivation of various growth factors. These processes have been shown to be regulated by p53; a tumor suppressor gene product.[8] Recently, it was reported that deprivation of isoleucine induced apoptosis in murine erythroleukemia cells, but not in mutant of these cells lacking wild-type p53 genes.[11]

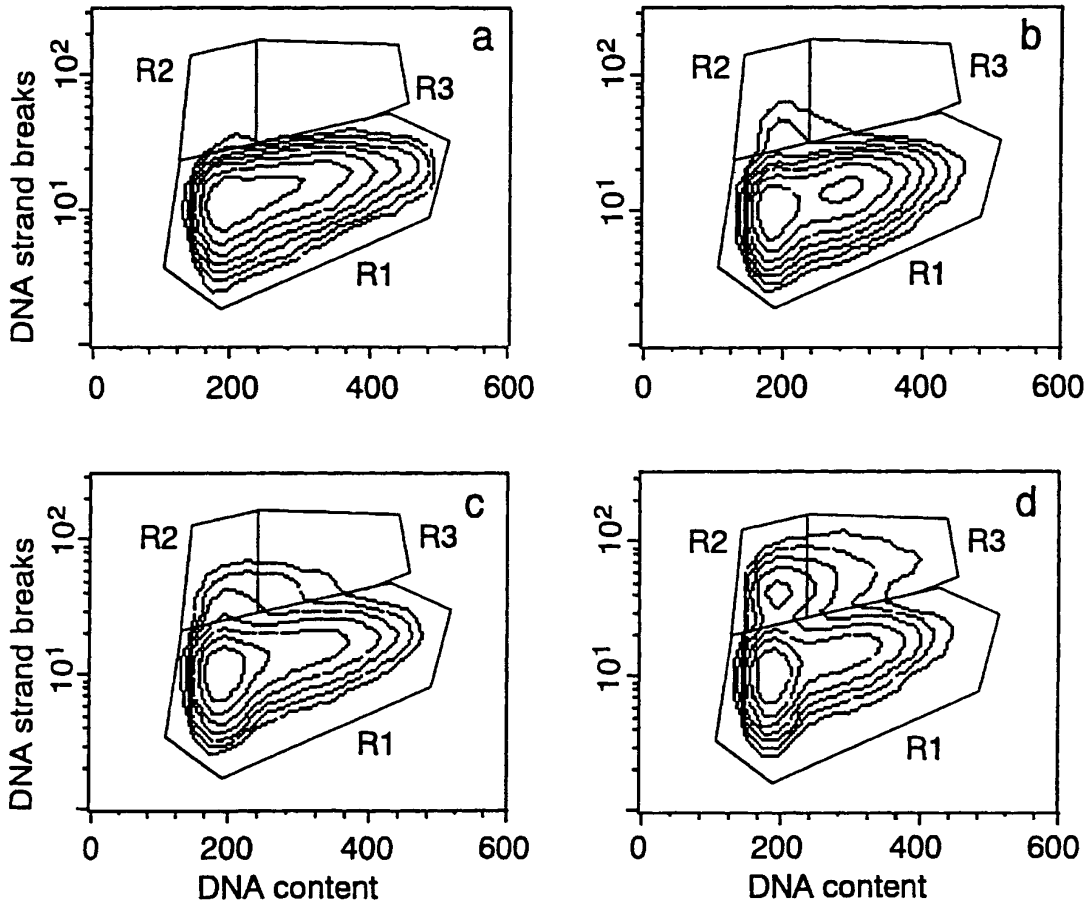

Figure 3 Two-color flow cytometry analysis for DNA strand breaks and cell cycle in L5178Y cells treated with L-asparaginase for (a) 0, (b) 8, (c) 16 and (d) 24 h. R1: cells without DNA strand breaks, R2: G1 cells with DNA strand breaks, R3: S and G2/M cells with DNA strand breaks. The percentage of cells in R1, R2 and R3 were: (a) 99.8%, 0.1%, 0.1%; (b) 98.6%, 1.1%, 0.3%; (c) 95.2%, 3.5%, 1.3%; (d) 85.4%, 11.3%, 3.3%.

Whether the apoptosis of leukemia cells induced by L-asparaginase depends on the function of wild-type p53 remains to be investigated.

In view of the present findings, we would suggest that the enzymes which degrade amino acids demand more attention as potential therapeutic agents. The problems associated with the therapy using exogenous enzymes have been the short clearance time in the circulation and immunogenicity. However, these problems were recently overcome by a new technology to modify enzymes with polyethylene glycol (PEG).[14,15] We reported that modification of L-asparaginase with PEG prolonged its blood circulation time,[16] diminished its immunoreactivity and immunogenicity,[17,18] and enhanced its antitumor activity.[19] The search for enzymes other than L-asparaginase that effectively block the external supply of certain amino acids may lead to the development of new agents that aid in the spontaneous induction of apoptosis in malignant cells.

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