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Acetylleucine Chloromethyl Ketone, an Inhibitor of Acylpeptide Hydrolase, Induces Apoptosis of U937 Cells

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Acetylleucine chloromethyl ketone (ALCK), an inhibitor of acylpeptide hydrolase (ACPH), inhibited the growth of human monoblastic U937 cells in a dose- and time-dependent manner. Morphology of the ALCK-exposed cells showed typical apoptosis, judging from the nuclear condensation and segmentation. Chromosomal DNA of U937 cells treated with ALCK showed an internucleosomal ladder-like pattern on electrophoresis, being characteristic of apoptosis.Of the other leucine chloromethyl ketone analogues, butyloylleucine chloromethyl ketone (BLCK) induced a weak ladder-like formation but caploylleucine chloromethyl ketone (CLCK)barely did. On the other hand, intracellular ACPH activity of U937 cells was strongly inhibited by culturing with ALCK, moderately with BLCK, and not with CLCK. These findings indicate that the inhibition of ACPH activity leads to apoptosis and suggest that ACPH may play a vital role in eukaryotic cells.

Apoptosis, a physiological controlled cell death (1, 2), is characterized by internucleosomal DNA fragmentation, chromatin condensation, and cytoplasmic blebbing. Apoptosis occurs to many types of cells during normal development (3) or in response to a variety stimuli, including DNA damage, growth factor deprivation, abnormal expression of oncogenes or tumor suppressor genes (4) and various chemicals including anti-cancer drugs (5–8). There apparently are a number of factors involved in the apoptotic process. Proteolytic enzymes such as caspase family and proteasome have been reported to promote or inhibit the transduction pathway to apoptotic cell death (9). But little argument have been presented about the other proteolytic enzymes.

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Acylpeptide hydrolase (ACPH, EC 3.4.19.1) catalyzes the hydrolysis of N-acylated peptide to an acylamino acid and a residual peptide (10). N-acylation is a common event in mammalian cells, in which 60-80% of intracellular proteins have been reported to be acetylated (11). But the cellular functions of ACPH and the significance of N-acylation have been unclear.

To study the role of ACPH on cellular functions, we examined the effect of ACPH inhibitors, acetylleucine chloromethyl ketone (ALCK) along with the other leucine chloromethyl ketone analogues, on the culture of human monoblastic U937 cells. We found that ALCK strongly induced the apoptotic cell death, which suggests that ACPH is one of the critical factors for living of cells.

MATERIALS AND METHODS

Cell lines and culture. Human myeloid leukemia U937 cell line was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. U937 cells were maintained in RPMI-1640 medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum and 60 μg/ml of kanamycin in a humidified atmosphere of 5% CO_2 and 95% air. Relative cell number was assessed by the MTT method (12).

Chemicals. ALCK was synthesized from L-leucine chloromethyl ketone · HCl (LCK, Sigma Chemical. Co., St. Louis, MO) (13). Butyloylleucine (BLCK) and caploylleucine chloromethyl ketone (CLCK) were also synthesized. Briefly, to a stirred and ice-cooled solution of 0.5 mmol LCK and 0.5 mmol butylic or caplonic anhydride in freshly distilled tetrahydrofuran, 0.5 mmol of triethylamine was added in a dropwise fashion. The mixture was stirred for 1 h at room temperature and purified by silica gel chromatography in chloroform. The spectral data of products were shown below. BLCK [Yield: 86.5%, colorless oil. CI-MS (*m/z*): 234 (MH^+, 100), HR-FAB (*m/z*): Calcd for $C_{11}H_{21}NO_2Cl$ (MH^+): 234.1260, Found: 234.1274], CLCK [Yield: 82.4%, colorless oil. CI-MS (*m/z*): 262 (MH^+,100), HR-FAB (*m/z*):Calcd for $C_{13}H_{25}NO_2Cl$ (MH^+): 262.1574, Found: 262.1554].

The substrate of ACPH (acethylalanine *p*-nitroanilide, AANA) and etoposide were obtained from Sigma Chemical.

ACPH assay. U937 cells were solubilized with PBS(−) containing 0.05% Triton X-100 and centrifuged at 13,000 g for 5 min. The supernatant (cell lysate) was assayed for ACPH activity using 0.3 mM AANA as substrate in 20 mM Tris-HCl (pH 7.4) (14). After

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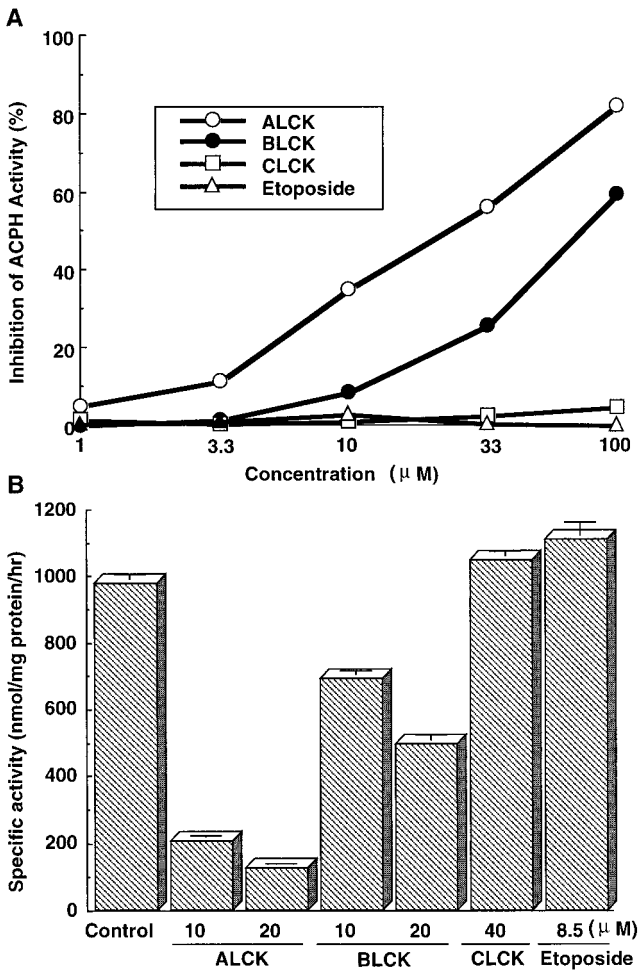

FIG. 1. Inhibition by ALCK, BLCK, and CLCK of ACPH activity in lysates (A) and in culture of U937 (B). (A) ACPH activity of U937 cell lysates was assayed in the presence of inhibitors. (B) After U937 cells were cultured with chemicals for 3 h, the cell lysates were prepared and assayed for ACPH activity as described in Materials and Methods. % inhibition = (1 − *p*-NA released in assay with inhibitor/*p*-NA released in assay with vehicle) × 100. Data are expressed as the mean ± S.E. (n = 4).

incubating at 37°C for 2 h, the liberated *p*-nitroaniline from AANA was measured at 405 nm using a Bio-Rad microplate reader, Model 3550. Protein content was determined by the Bradford method (15).

DNA fragmentation assay. After culturing U937 cells (2×10^5) with 20 μM ALCK, BLCK, CLCK, or 8.5 μM etoposide, they were harvested and suspended in 20 μl of 50 mM Tris-HCl buffer (pH 8.0), 10 mM EDTA and 0.5 mg/ml proteinase K (Wako Pure Chemicals), and incubated at 50°C for 1 h. Then, 10 μl of RNase A (Funakoshi Co., Tokyo) solution was added and incubated for an additional time. The samples were mixed with pre-heated low-melting point agarose (Wako Pure Chemicals), analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide, and then photographed on a UV illuminator (16).

RESULTS AND DISCUSSION

Inhibition of ACPH activity by ALCK, BLCK, and CLCK. First we tested the inhibitory effects of ALCK, BLCK, or CLCK on ACPH activity of U937 cell lysate using AANA as a substrate. The ACPH activity was strongly inhibited by ALCK (13), moderately by BLCK, and marginally by CLCK. The apparent IC_{50}s of ALCK and BLCK were 15 μM and 50 μM, respectively (Fig. 1A). The IC_{50}s against ACPH purified from human peripheral blood leukocytes were 7.5 μM and 30 μM, respectively (data not shown). A topoisomerase II inhibitor etoposide, which was well known to induce apoptosis of many type of cells, did not inhibit ACPH activity in the dose range which might induce apoptosis. Secondly, we examined the inhibition of ACPH activity in U937 cells by ALCK, BLCK, or CLCK. After culturing U937 cells in the presence of each compound for 3 h, the cells were intensively washed, the cell lysates were prepared, and assayed for ACPH activity. The ACPH activity was decreased to 21.0% of control by 10 μM ALCK and 70.8% by 10 μM BLCK, respectively, but not by 40 μM CLCK (Fig. 1B). The observed inhibition to the intracellular ACPH activity by the LCK-derivatives was similar to that to the ACPH activity of normal cell lysates as shown in Fig. 1A.

Inhibition by ALCK of U937 cell growth. We tested whether the inhibition of ACPH resulted in suppression of cell growth. U937 cells were cultured in the presence of 5 to 40 μM ALCK for 20 h. Fifty percent inhibition was observed at about 12 h in the presence of 20 μM ALCK or 8.5 μM etoposide (Fig. 2).

Morphological changes in the nuclei of U937 cells by ALCK treatment. We noticed the apoptosis-like cell death of U937 cells by ALCK under a phase contrast microscopy during growth inhibition test. U937 cells cultured with 40 μM ALCK showed typical apoptotic figures (Fig. 3C). Etoposide induced marked nuclear morphological changes of U937 cells at 8.5 μM (Fig. 3D).

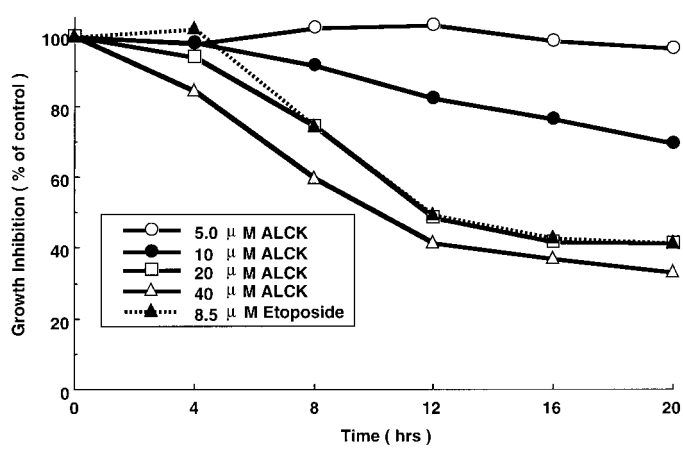

FIG. 2. Growth inhibition of U937 cells by ALCK. U937 cells were cultured with 5, 10, 20, or 40 μM ALCK, or 8.5 μM etoposide. The number of viable cells was determined by the MTT method. % of control = viable cells in culture with inhibitor/viable cells in culture with vehicle alone 100. Data are expressed as the mean of four cultures.

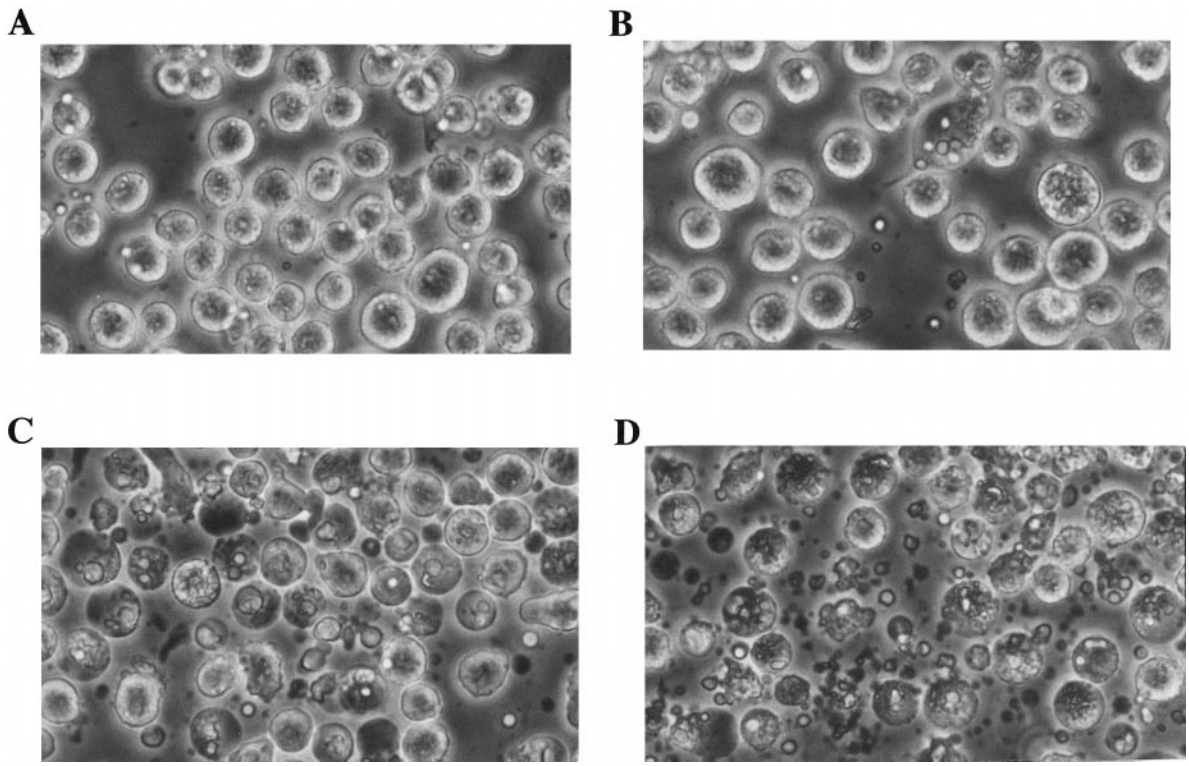

FIG. 3. Morphology of U937 cells cultured with ALCK. U937 cells were cultured for 12 h with vehicle alone (A), 10 (B), and 40 μM ALCK (C), or 8.5 μM etoposide (D), and then observed under a phase-contrast microscopy (×400).

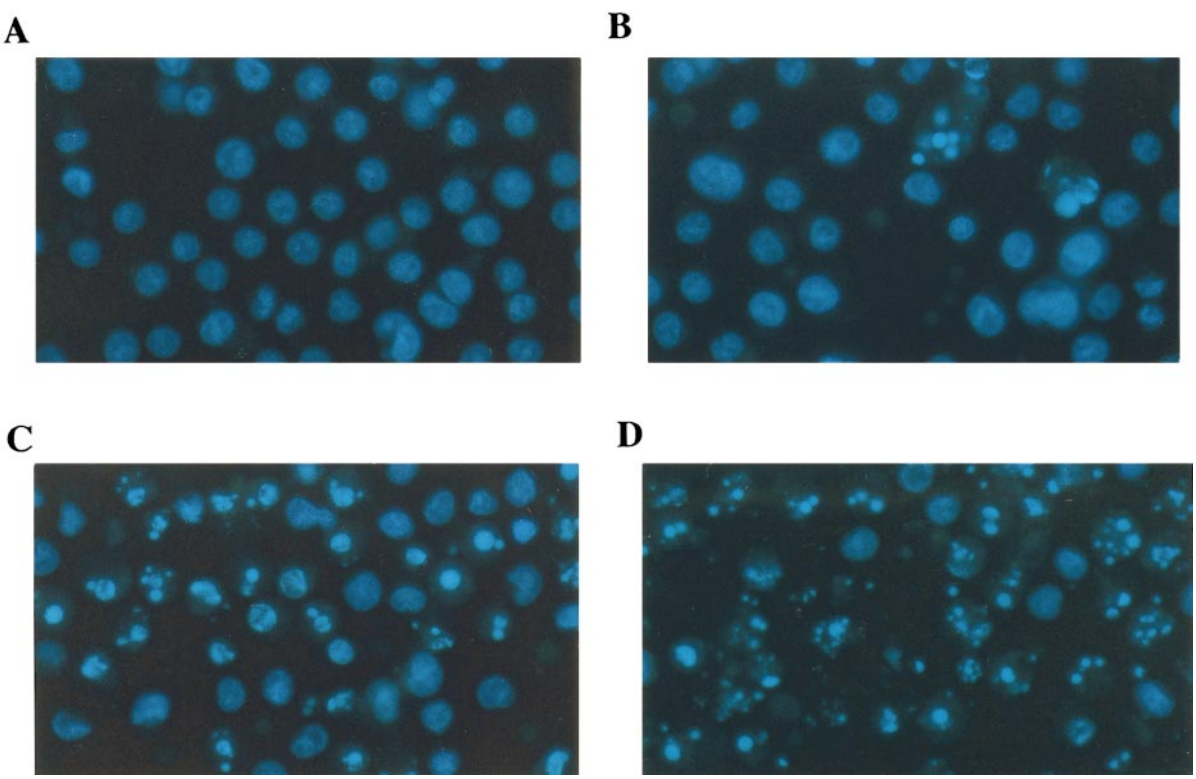

FIG. 4. Micrographs of Hoechst 33342-stained U937 cells treated with ALCK. U937 cells were cultured for 12 h with vehicle alone (A), 10 (B), and 40 μM ALCK (C), or 8.5 μM etoposide (D). Cells were stained with Hoechst33342 and observed under a fluorescence microscopy (×400).

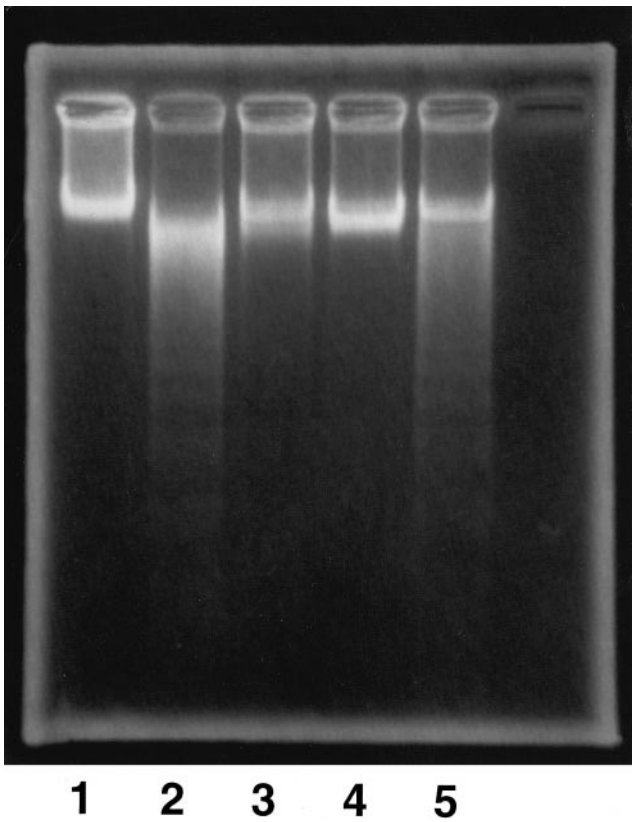

FIG. 5. Oligonucleosomal DNA fragmentation of U937 cells treated with ALCK. U937 cells were cultured for 12 h with vehicle alone (lane 1), 20 μM ALCK (lane 2), 20 μM BLCK (lane 3), 40 μM CLCK (lane 4), and 8.5 μM etoposide (lane 5). DNA fragments were separated on a 2.0% agarose gel and stained with ethidium bromide.

The cells in the same view of Fig. 3 were stained with an apoptosis detecting agent Hoechst 33342 solution (Fig. 4). The nuclear condensation and segmentation characteristic of apoptotic changes were obviously demonstrated in U937 cells treated with 40 μM ALCK (Fig. 4C), which were similar to these in the cells treated with 8.5 μM etoposide (Fig. 4D).

Oligonucleosomal fragmentation of U937 cells by ALCK. In addition to the morphological study, we tested whether DNA ladder formation, a critical biochemical marker for apoptosis were induced by ALCK, BLCK, or CLCK. As shown in Fig. 5, oligonucleosomal fragmentations were clearly observed by culturing U937 cells at 20 μM ALCK (lane 2) and a little observed at 20 μM BLCK (lane 3), but not all even at 40 μM CLCK (lane 4). Etoposide gave a typical DNA ladder at 8.5 μM (lane 5). It was suggested that the loss of ACPH activity in cells resulted in apoptosis of U937 cells.

Together with all data obtained in the present the study, it was concluded that the inhibition of ACPH activity led the apoptosis in U937 cells. It remains unknown whether some members of caspase family (17) are involved in the apoptotic process triggered by ALCK. The present data suggest that deacylation of acylpeptide might be one of the requisites for cell survival.

REFERENCES

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) *Br. J. Cancer* **26,** 239–257.
2. Wyllie, A. H. (1980) *Nature* **284,** 555–556.
3. Robley, E., and Fowlkes, B. J. (1994) *Annu. Rev. Immunol.* **12,** 675–705.
4. Levine, A. J. (1997) *Cell* **88,** 323–331.
5. Kaufmann, S. H. (1989) *Cancer Res.* **49,** 5870–5878.
6. Bino, D. G., Bruo, S., Yi, P. N., and Darzynkiewicz, Z. (1992) *Cell Prolif.* **25,** 537–548.
7. Gunji, H., Kharbanda, S., and Kufe, D. (1991) *Cancer Res.* **51,** 741–743.
8. Evans, D. L., and Dive, C. (1993) *Cancer Res.* **53,** 2133–2139.
9. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) *Cell* **87,** 171.
10. Tsunasawa, S., Narita, K., and Ogata, K. (1978) *J. Biochem. (Tokyo)* **77,** 89–102.
11. Brown, J. L. (1979) *J. Biol. Chem.* **254,** 1447–1449.
12. Hansen, M. B., Nielsen, S. E., and Berg, K. (1989) *J. Immunol. Methods* **119,** 203–210.
13. Scaloni, A., Jones, W. M., Barra, D., Pospischil, M., Sassa, S., Popowicz, A., Manning, L. R., Schneewind, O., and Manning, J. M. (1992) *J. Biol. Chem.* **267,** 3811–3818.
14. Sharma, K. K., and Ortwerth, B. J. (1993) *Eur. J. Biochem.* **216,** 631–637.
15. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
16. Mashima, T., Naito, M., Kataoka, S., Kawai, H., and Tsuruo, T. (1995) *Biochem. Biophys. Res. Commun.* **209,** 907–915.
17. Cohen, G. M. (1997) *Biochem. J.* **326,** 1–16.