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# Immunocytochemistry of Apoptosis Induced by Bromodeoxyuridine in Human Leukemic HL-60 Cells

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**Various antitumor drugs which target tumor cells in the S-phase of the cell cycle induce apoptosis in HL-60 cells. The present study shows that apoptosis is inducible in HL-60 cells by continuous exposure to bromodeoxyuridine (BrdU), an S-phase specific reagent and a thymidine analogue. The localization of BrdU in the apoptotic cells was visualized with immunogold particles using an anti-BrdU monoclonal antibody. After 3 days of treatment with 20 $\mu M$ BrdU, cells ceased to grow and their viability was significantly decreased. Morphologically, nuclear segmentation and cytoplasmic maturation were observed in many viable cells, and the dead cells showed typical features of apoptosis. The nuclear DNA of apoptotic cells was visualized by the immunocytochemical detection of BrdU with post-embedding immunogold staining. The condensed chromatin of the apoptotic cells was highly labeled with immunogold particles, while the nucleolus was sparsely labeled. The chromatin could be traced with BrdU-immunocytochemistry even after disappearance of the nuclear envelope. These results indicated that apoptosis in HL-60 cells was induced by incorporation of BrdU into DNA of the S-phase cells. Using BrdU-immunocytochemistry, the localization of nuclear DNA in apoptotic cells was visualized and the changes of nuclear structures were followed during the progression of apoptosis.**

Key words: Apoptosis — Bromodeoxyuridine — Condensed chromatin — HL-60 cells — Immunogold labeling

Bromodeoxyuridine (BrdU), a synthetic analogue of thymidine which can be incorporated into DNA during the S-phase of the cell cycle, has been employed for immunohistochemical studies of cell proliferation using antibodies against BrdU.[1,2] Continuous delivery of BrdU by an osmotic minipump enables long-term analysis of the kinetics of cell response to various stimuli. However, the effects of BrdU incorporation into DNA on the host cells have not been well established.

Human leukemic HL-60 cells have been extensively studied as a model of the control of proliferation and differentiation.[3] Also, apoptosis induced in HL-60 cells by various chemical stimuli has been studied.[4–6] Terminal differentiation of HL-60 cells occurs over a period of two division cycles, during which the inducer must be continuously present.[7] This process consists of early and late events, the early event (defined as precommitment) occurring over the first division cycle, and the late event (defined as onset of terminal differentiation) occurring over the second division cycle.[8–10] Although BrdU treatment causes cell differentiation in some cell lines,[11,12] this compound induces the early event leading to precommitment, but not the late event leading to onset of terminal differentiation in HL-60 cells.[13]

In this report, we show that apoptosis in HL-60 cells could be induced by incorporation of BrdU into the S-phase cell nuclei. Therefore, the immunoelectron microscopy of BrdU can be used to visualize the localization of nuclear DNA in apoptotic cells, and thus to monitor the progression of apoptosis of HL-60 cells.

## MATERIALS AND METHODS

**Cell culture** The HL-60 cells were maintained in constant exponential growth using RPMI 1640 (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 m$M$ L-glutamine at 37°C in a 95% humidified atmosphere of 5% $CO_2$. The cells were passaged every third day, and the cell density in cultures did not exceed $5\times10^5$ cells/ml. The initial cell density of experimental cultures was $1\times10^5$/ml. BrdU (Sigma Chemical Co., St. Louis, MO) was added from a 20 m$M$ stock of BrdU in distilled water to give a final concentration of 20 $\mu M$. Control cultures were treated with distilled water at a final concentration of 0.1% in culture medium. Cell density was assayed with a hemocytometer. Cell viability was determined by the trypan blue dye exclusion method.

**Electron microscopy** The harvested cells were fixed with 2% glutaraldehyde in 0.1 $M$ cacodylate buffer (pH 7.4) at room temperature for 2 h. The cells were rinsed with phosphate-buffered saline (PBS) containing 0.1 $M$ lysine, embedded in 2% agarose, then postfixed with 1% osmium tetroxide in 0.1 $M$ cacodylate buffer (pH 7.4) containing 1.5% potassium ferrocyanide (reduced osmium)

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at 4°C for 1 h. The specimens were dehydrated in cold graded ethanol solutions and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections on nickel grids were etched with 10% $H_2O_2$ for 10 min followed by digestion with 0.2% trypsin in 0.05 *M* Tris/HCl buffer containing 0.1% $CaCl_2$ at pH 7.6 for 30 min at 37°C.[14,15] These pretreated thin sections were incubated on a drop of PBS containing 10% normal goat serum, 1% bovine serum albumin (BSA), and 0.1% Tween 20 (washing buffer) for 1 h, and then incubated with monoclonal anti-BrdU antibody (Becton Dickinson and Co., Mountain View, CA) diluted 1/100 with washing buffer for 3–4 h at room temperature. After four washes (floating on washing buffer) for 5 min each, the thin sections were incubated with goat anti-mouse IgG+IgM linked to 10-nm colloidal gold particles (BioCell Research Laboratories, Cardiff, UK) diluted 1/50 with BSA-PBST for 1 h at room temperature. The sections were then floated four times on washing buffer and three times on distilled water for 5 min each. After drying, the sections were stained with uranyl acetate and lead citrate. For controls, the sections were reacted only with secondary antibody or with non-immune mouse serum instead of the primary antibody.

## RESULTS

Exposure of HL-60 cells to 20 $\mu M$ BrdU for 5 days decreased the cell number to approximately 50% of the yield of control cultures (Fig. 1A), while nonviable cells were increased to 44% (Fig. 1B). The untreated HL-60 cells were typical promyelocytes with a high nuclear/cytoplasmic ratio and a large central round nucleus having prominent nucleoli (Fig. 2A). After 3 days of treatment with BrdU, approximately 60% of viable cells showed nuclear segmentation with many cytoplasmic vacuoles (Fig. 2C). Nonviable cells showed typical morphology of apoptotic cells. Condensed chromatin at the nuclear periphery was observed in the apoptotic cells in which the nuclear envelope was retained (Fig. 3A). In progressed apoptotic cells, multiple condensed chromatin spheres were observed (Fig. 4A) and the nuclear envelopes had disappeared. Both viable and apoptotic cells in the post-embedding preparation were positive for BrdU with the immunogold technique. The condensed chromatin was highly labeled with immunogold particles, while the nucleolus was only sparsely labeled (Figs. 3B, 4B, 4C, and 4D).

## DISCUSSION

In this study, we demonstrated that continuous exposure of HL-60 cells to BrdU arrested cell growth and increased the number of apoptotic cells. Nuclear segmentation and cytoplasmic maturation were seen in viable cells. As these changes were marked after 3 days of treatment of HL-60 cells with BrdU, the continuous state of precommitment induced by BrdU might result in the induction of apoptosis. Many antitumor drugs commonly induce apoptosis by targeting tumor cells in the S-phase of the cell cycle.[16] BrdU, a thymidine analogue, is an S-phase specific reagent which is incorporated into nuclei during the S-phase.[1,2] 5-Fluorodeoxyuridine (FdUrd), which is similar in structure to BrdU, is a derivative of 5-fluorouracil (5-FU), a commercially available anti-tumor drug. FdUrd incorporation into DNA can produce lethal cellular events.[17] In some tumor cell lines, FdUrd leads to an unbalanced pool of deoxyribonucleotides and generates a significant number of double-strand DNA breaks.[18–20] BrdU, therefore, may have similar effects to those of FdUrd. In fact, immunogold particles of BrdU were localized in the viable cell nuclei and in the con-

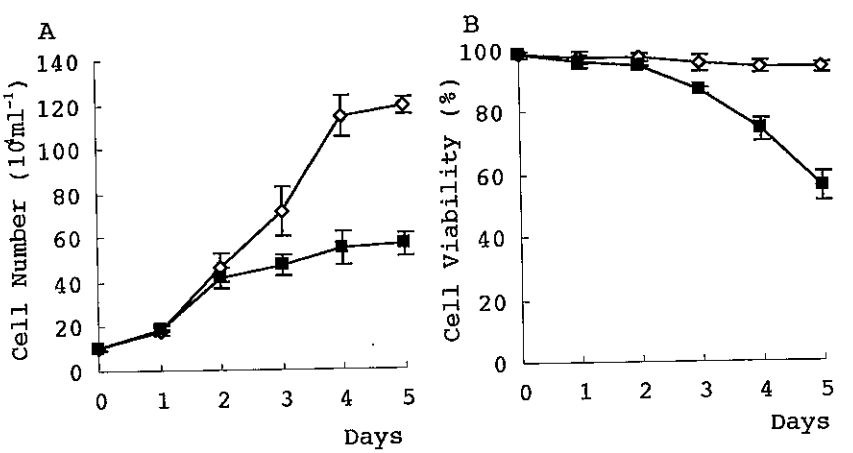


Fig. 1. Effects of BrdU on the growth (A) and viability (B) of HL-60 cells. HL-60 cells were continuously exposed to 20 $\mu M$ BrdU or vehicle (control). ◇ control; ■ BrdU.

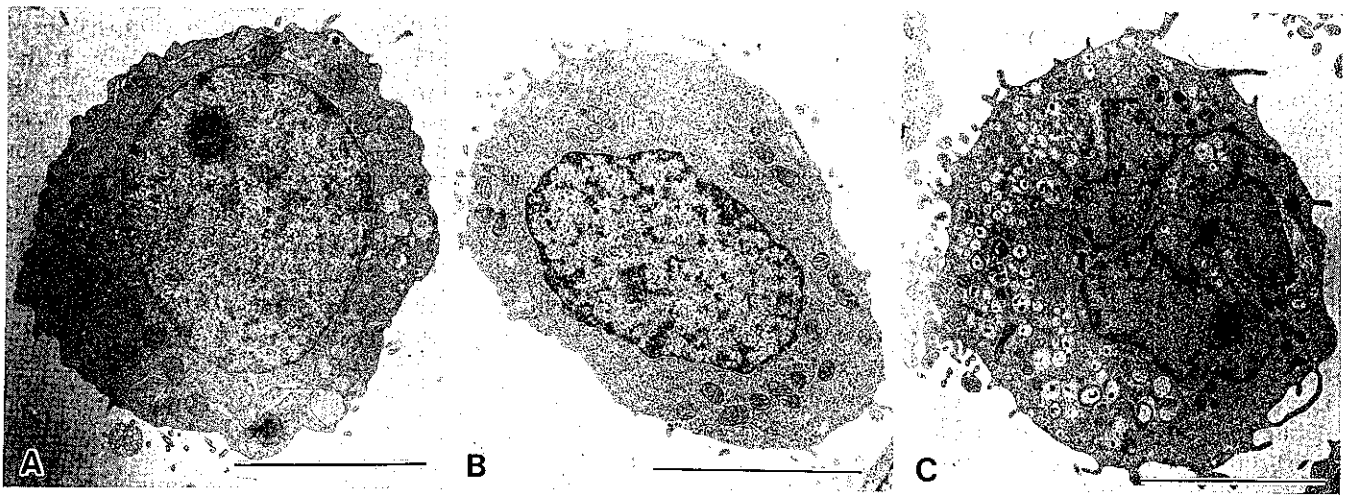


Fig. 2. Morphological change of HL-60 cells induced by 20 $\mu M$ BrdU treatment. A, The untreated HL-60 cell is a typical promyelocyte. ×7,100. Bar=5 $\mu$m; A pair of transmission electron micrographs of HL-60 cells after treatment with 20 $\mu M$ BrdU for 3 days. B, An HL-60 cell with a normal nucleus. ×7,800. Bar=5 $\mu$m. C, Nuclear segmentation and cytoplasmic maturation with many vacuoles in an HL-60 cell. ×6,800. Bar=5 $\mu$m.

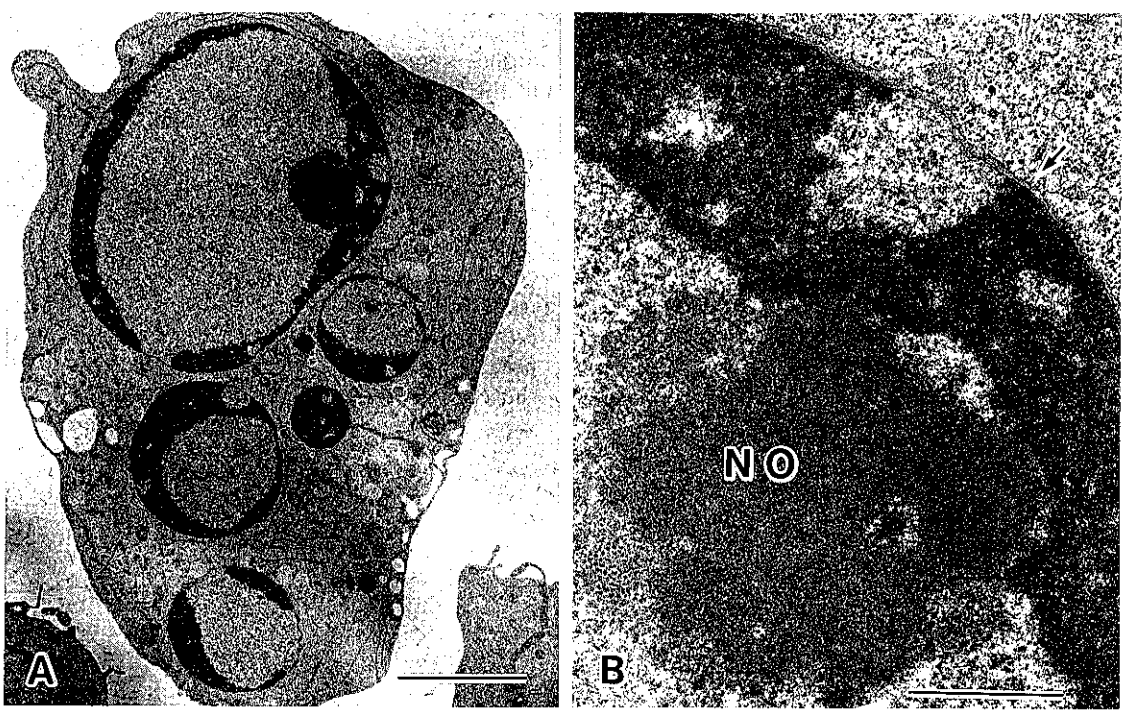


Fig. 3. A, The condensed chromatin at the nuclear periphery in apoptotic cells after treatment of HL-60 cells with 20 $\mu M$ BrdU for 3 days. At low magnification. ×3,800. Bar=5 $\mu$m. B, A part of Fig. 3A at higher magnification. The condensed chromatin is highly labeled with immunogold particles (BrdU), while the nucleolus (NO) is sparsely labeled. An arrow indicates the nuclear envelope. ×22,800. Bar=1 $\mu$m.

densed chromatin of apoptotic cells. Cytotoxicity of BrdU to HL-60 cells may result from the inhibition of DNA metabolism due to the incorporation of BrdU into nuclei of the S-phase cells. BrdU inhibited cell growth and induced apoptosis in HL-60 cells. These effects are similar to those of antitumor drugs targeting S-phase cells.

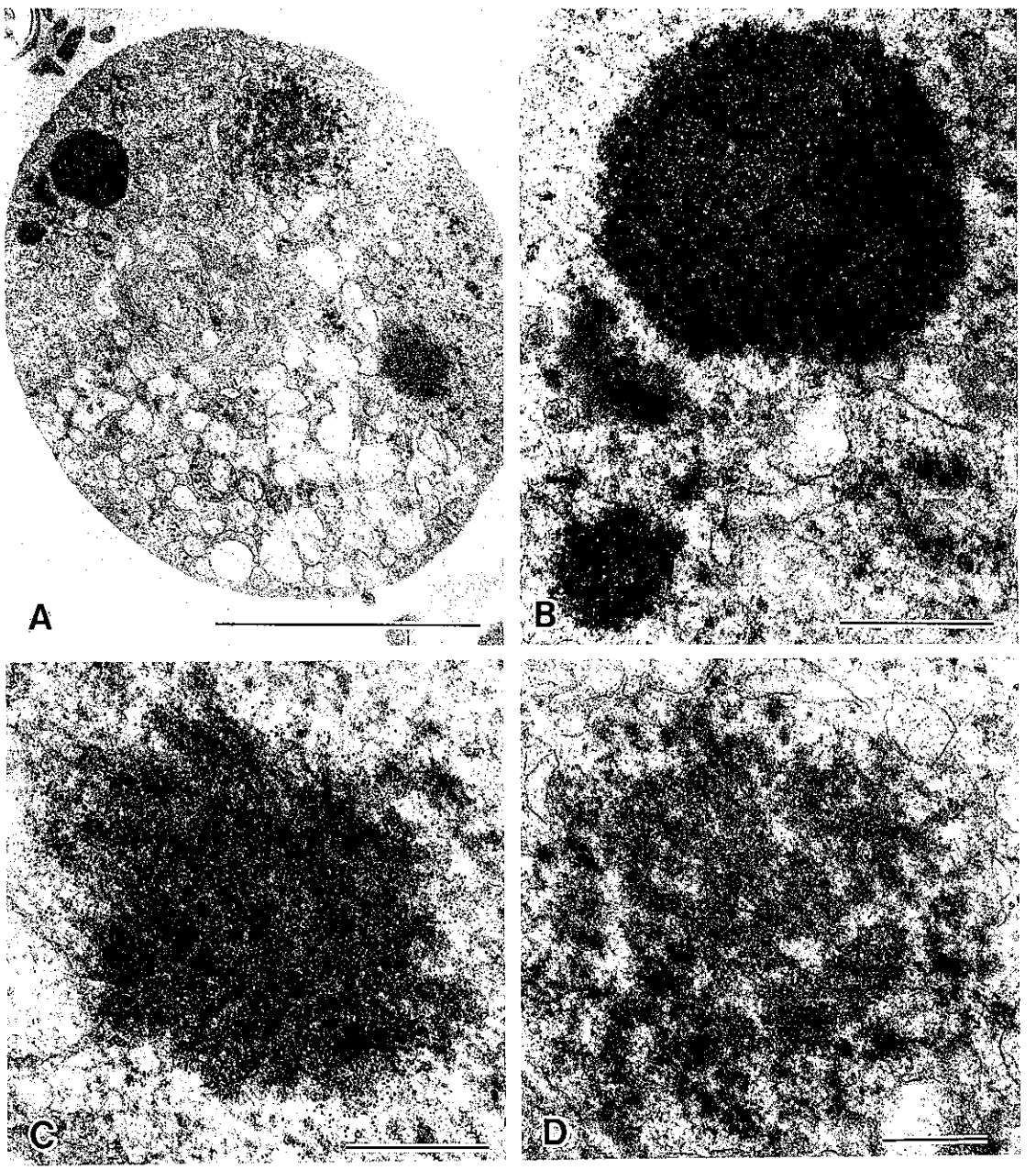


Fig. 4. In advanced apoptosis, the multiple condensed chromatin spheres are not surrounded by nuclear envelopes. Low (A) and high (B, C, D) magnification. The condensed chromatin spheres are highly labeled with immunogold particles (B and C), while the nucleolus is sparsely labeled (D). A, ×8,500. Bar=5 μm; B, ×47,000. Bar=0.5 μm; C, ×42,500. Bar=0.5 μm; D, ×32,500. Bar=0.5 μm.

All cell nuclei were highly labeled with immunogold particles. In the apoptotic cells, the condensed chromatin and nucleolus were separately located. At the electron microscopic level, the condensed chromatin and nucleolus could be distinguished in apoptotic cells while their nuclear envelopes were still retained. However, the nuclear structure became multispheric and the nuclear envelope disappeared with the progression of apoptosis. In such apoptotic cells, it was difficult to distinguish chromatin from the nucleolus by conventional electron microscopy. In this study, the condensed chromatin was highly labeled with immunogold particles, while the nu-

cleolus was sparsely labeled. Thus, BrdU-immunocytochemistry can distinguish the chromatin. The sparse localization of BrdU in the nucleolus reflects intranucleolar DNA.[21] The presence of condensed chromatin at the nuclear periphery and multiple chromatin spheres in apoptotic cells is well known.[22, 23] However, the localization of nuclear DNA in these apoptotic cells has not been well visualized. Using the present BrdU-immunocytochemistry, with a high resolution of fine structure due to the conventional electron microscopic preparation,[7, 8] we could visualize the localization of nuclear DNA in apoptotic cells of HL-60 cells and could monitor the changes of nuclear structures during the progression of apoptosis even after the nuclear envelopes had disappeared in HL-60 cells.

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REFERENCES


1) Gratzner, H. G. Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science,* **218,** 474–475 (1982).
2) Dolbeare, R., Gratzner, H., Pallavicini, M. G. and Gray, J. W. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA,* **80,** 5573–5577 (1983).
3) Collins, S. J. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood,* **70,** 1233–1238 (1987).
4) Martin, S. J., Bradley, J. G. and Cotter, T. G. HL-60 cells induced to differentiate toward neutrophils subsequently die via apoptosis. *Clin. Exp. Immunol.,* **79,** 448–453 (1990).
5) Solary, E., Bertrand, R., Kohn, K. W. and Pommier, Y. Differential induction of apoptosis in undifferentiated and differentiated HL-60 cells by DNA topoisomerase I and II inhibitors. *Blood,* **81,** 1359–1368 (1993).
6) Solary, E., Bertrand, R. and Pommier, Y. Apoptosis of human leukemic HL-60 cells induced to differentiate by phorbol ester treatment. *Leukemia,* **8,** 792–797 (1994).
7) Yen, A. Control of HL-60 myeloid differentiation: evidence of uncoupled growth and differentiation control, S-phase specificity and two-step regulation. *Exp. Cell Res.,* **156,** 198–212 (1984).
8) Yen, A., Reece, S. L. and Albright, K. L. Dependence of HL-60 myeloid cell differentiation on continuous and split retinoic acid exposures: pre-commitment memory associated with altered nuclear structure. *J. Cell. Physiol.,* **118,** 277–286 (1984).
9) Momoi, T., Seno, H. and Yoshikura, H. Two stages of the differentiation of HL-60 cells induced by 1$\alpha$,25-dihydroxyvitamin $D_3$ and promotion by DMSO. *Biosci. Rep.,* **6,** 96–101 (1986).
10) Yen, A., Forbes, M., deDala, G. and Fishbaugh, J. Control of HL-60 cell differentiation lineage specificity: a late event occurring after precommitment. *Cancer Res.,* **47,** 129–134 (1987).
11) Sugimoto, T., Kato, T., Sawada, T., Horii, Y., Kemshead, J. T., Hino, T., Morioka, H. and Hosoi, H. Schwannian cell differentiation of human neuroblastoma cell lines *in vitro* induced by bromodeoxyuridine. *Cancer Res.,* **48,** 2531–2537 (1988).
12) Saito, Y., Saido, T. C., Sano, K. and Kawashima, S. The calpain-calpastatin system is regulated differently during human neuroblastoma cell differentiation to Schwannian and neuronal cells. *FEBS Lett.,* **353,** 327–331 (1994).
13) Yen, A. and Forbes, M. E. c-*myc* Down regulation and precommitment in HL-60 cells due to bromodeoxyuridine. *Cancer Res.,* **50,** 1411–1420 (1990).
14) Kondo, K. and Makita, T. Detection of bromodeoxyuridine (BrdU) in semi-thin sections of routine electron microscopic specimens digested with trypsin. *J. Vet. Med. Sci.,* **58,** 583–585 (1996).
15) Kondo, K. and Makita, T. Electron microscopic immunogold labeling of bromodeoxyuridine (BrdU) in routine electron microscopy. *Acta Histochem. Cytochem.,* **29,** 115–119 (1996).
16) Gorczyca, W., Gong, J., Ardelt, B., Traganos, F. and Darzynkiewicz, Z. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res.,* **53,** 3186–3192 (1993).
17) Kufe, D. W., Major, P. P., Egan, E. M. and Loh, E. 5-Fluoro-2′-deoxyuridine incorporation into L1210 DNA. *J. Biol. Chem.,* **256,** 8885–8888 (1981).
18) Yoshioka, A., Tanaka, K., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C. and Wataya, Y. Deoxyribonucleoside-triphoshate imbalance: 5-fluorodeoxyuridine-induced DNA double-strand breaks in mouse FM3A cells and the mechanism of cell death. *J. Biol. Chem.,* **262,** 8235–8241 (1987).
19) Ayusawa, D., Arai, H., Wataya, Y. and Seno, T. A specialized form of chromosomal DNA degradation induced by thymidylate stress in mouse FM3A cells. *Mutat. Res.,* **200,** 221–230 (1988).
20) Yin, M. B. and Rustum, Y. M. Comparative DNA strand breakage induced by Fura and FdUrd in human ileocecal adenocarcinoma (HCT-8) cells: relevance to cell growth inhibition. *Cancer Commum.,* **3,** 41–51 (1991).
21) Thiry, M. Immunoelectron microscopic localization of bromodeoxyuridine incorporated into DNA of Ehrlich tumor cell nucleoli. *Exp. Cell Res.,* **179,** 204–213 (1988).
22) Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. Cell death: the significance of apoptosis. *Int. Rev. Cytol.,* **68,** 251–305 (1980).
23) Arends, M. J. and Wyllie, A. H. Apoptosis: mechanisms and roles in pathology. *Int. Rev. Exp. Pathol.,* **32,** 223–254 (1991).