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Features of DNA Oligonucleosomal Fragmentation in Human Tumor Cell Lines and Its Detection by Flow Cytometry: Utility and Limitations

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Cultured HL-60, HeLa S3 and WiDr cells were treated with various doses of ethanol, then subjected to flow cytometry and gel electrophoresis of cellular DNA. On electrophoresis of DNA from HL-60 cells treated with 0.5 or 1.0 m*M* ethanol, a ladder pattern was recognized after 3 h. At higher doses of ethanol (2.0 and 5.0 m*M*), a smear pattern resulted. On flow cytometry, however, A_0 cells (lower fluorescence level than G_0+G_1 cells) were noted from 0.5 to 5.0 m*M* ethanol. The observation of A_0 cells at higher doses indicated loss of DNA after random DNA degradation. HeLa S3 and WiDr cells were partially detached from flasks after administration of ethanol and separated into adherent and non-adherent categories. In DNA from non-adherent HeLa S3 cells treated with 0.5 m*M* ethanol, a ladder pattern was observed after 24 h. On flow cytometry, prior to the appearance of A_0 cells, an accumulation in the G_2+M-phase became obvious after 3 h. Increased mitotic indices indicated that this phenomenon was due to M-phase arrest. Adherent HeLa S3 cells showed no DNA oligonucleosomal fragmentation or A_0 cells. These findings indicate that detection of A_0 cells by flow cytometry is not proof of cell death by DNA oligonucleosomal fragmentation.

Key words: DNA oligonucleosomal fragmentation — Cell death — DNA gel electrophoresis — Flow cytometry

A number of approaches have been adopted to distinguish apoptotic cells from living or necrotic cells.[1–5] At present, apoptosis is not determined only by the presence of DNA oligonucleosomal fragmentation.[6–8] Darzynkiewicz *et al.*[2] have reported the usefulness of flow cytometry for detecting apoptotic cells as so-called A_0 cells in DNA histograms. In apoptosis, DNA is presumed to be cut into lengths that are integral multiples of 180–200 base pairs in nucleosomes, this being responsible for the ladder pattern observed on gel electrophoresis.[9] Low-molecular-weight DNA is likely to be lost from apoptotic cells, resulting in a lower DNA content as compared non-apoptotic cells, so that this population forms a peak of A_0 cells.[2] This peak, however, only represents a population that contains a diminished amount of DNA, and does not necessarily indicate that DNA oligonucleosomal fragmentation has occurred.

In the present study, we compared flow cytometry with gel electrophoresis of cellular DNA to determine whether the former approach is valid for investigation of DNA oligonucleosomal fragmentation. Tumor cell lines were treated with ethanol to induce cell death and processed for both flow cytometrical and molecular biological studies. Differences between adherent and non-adherent tumor cells were also investigated.

MATERIALS AND METHODS

Cell lines and culture conditions HeLa S3, WiDr and HL-60 cells were purchased from Dai-Nippon Seiyaku Co., Ltd., Tokyo and maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo) or RPMI1640 medium (Nissui) for HL-60, both containing penicillin G (5×10^3 U/ml, Gibco BRL, Gaithersburg, MD), streptomycin (5 mg/ml, Gibco BRL) and 10% fetal bovine serum (Bioserum, Victoria). Cells were cultured at 37°C under 5% CO_2 in a fully humidified atmosphere.

Electrophoresis of cellular DNA Some HeLa S3 and WiDr cells became detached from culture flasks after treatment with ethanol. These cells were defined as non-adherent and the remainder as adherent in the present study. Cells were collected by centrifugation, washed twice in ice-cold Tris-buffered saline, and resuspended in 500 μl of lysis buffer containing 500 m*M* Tris-HCl (pH 9.0), 2 m*M* EDTA, 10 m*M* NaCl, 1% SDS and 1 mg/ml proteinase K (Wako Chemical, Osaka). After incubation at 50°C for 20 h, samples were extracted twice with phenol, once with phenol-chloroform and finally with chloroform. Each sample was loaded on a 1.2% agarose (Nippon Gene, Tokyo) gel. Electrophoresis was performed for 70 min at 7 V/cm in Tris-acetate-EDTA buffer. Gels were stained with 0.5 μg/ml ethidium bromide (15 min) and photographed under UV (254 nm).

Flow cytometry After culture, cells were washed twice with ice-cold PBS and fixed in 70% ethanol (−20°C) overnight. Samples were centrifuged and incubated for one hour at room temperature in PBS containing 0.1% Triton-X[16] followed by staining with propidium iodide (50 μg/ml, Sigma, St. Louis, MO). The DNA content of 2×10^4 cells was measured using a cell sorter (EPICS-ELITE, Coulter Electronics, Hialeah, FL) and DNA distribution histograms were obtained.

Colony formation assay Cells treated with or without ethanol were washed with ice-cold PBS and counted. Samples of one hundred HeLa S3 cells were seeded onto Petri dishes (Falcon 3002, Becton Dickinson & Co., Lincoln Park, NJ) and cultured for 14 days. After culture, dishes were washed twice with PBS followed by methanol fixation and Giemsa staining, and colonies were counted.

Mitotic index HL-60 and HeLa S3 cells were washed with ice-cold PBS and spread on slide glasses followed by May-Giemsa staining. Numbers of mitotic-phase cells were counted in samples of 500 cells.

RESULTS

Gel electrophoresis of cellular DNA Cellular DNA extracted from HL-60 cells showed a typical ladder pattern after a 3 h exposure to 0.5 m*M* ethanol. At 1.0 m*M*, a ladder pattern was seen until 6 h after the exposure, although the cellular DNA showed a smear pattern[8] at 12 and 24 h. At greater doses of ethanol, no ladder

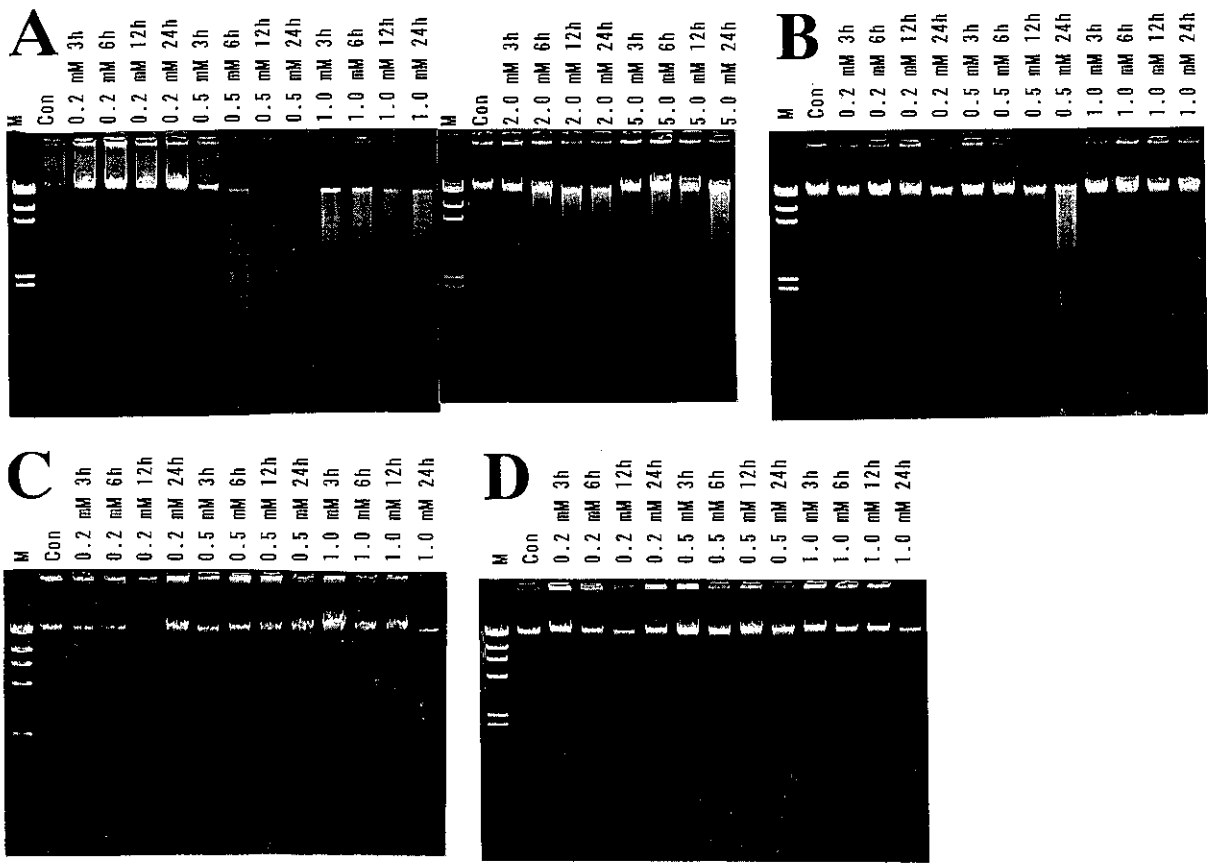
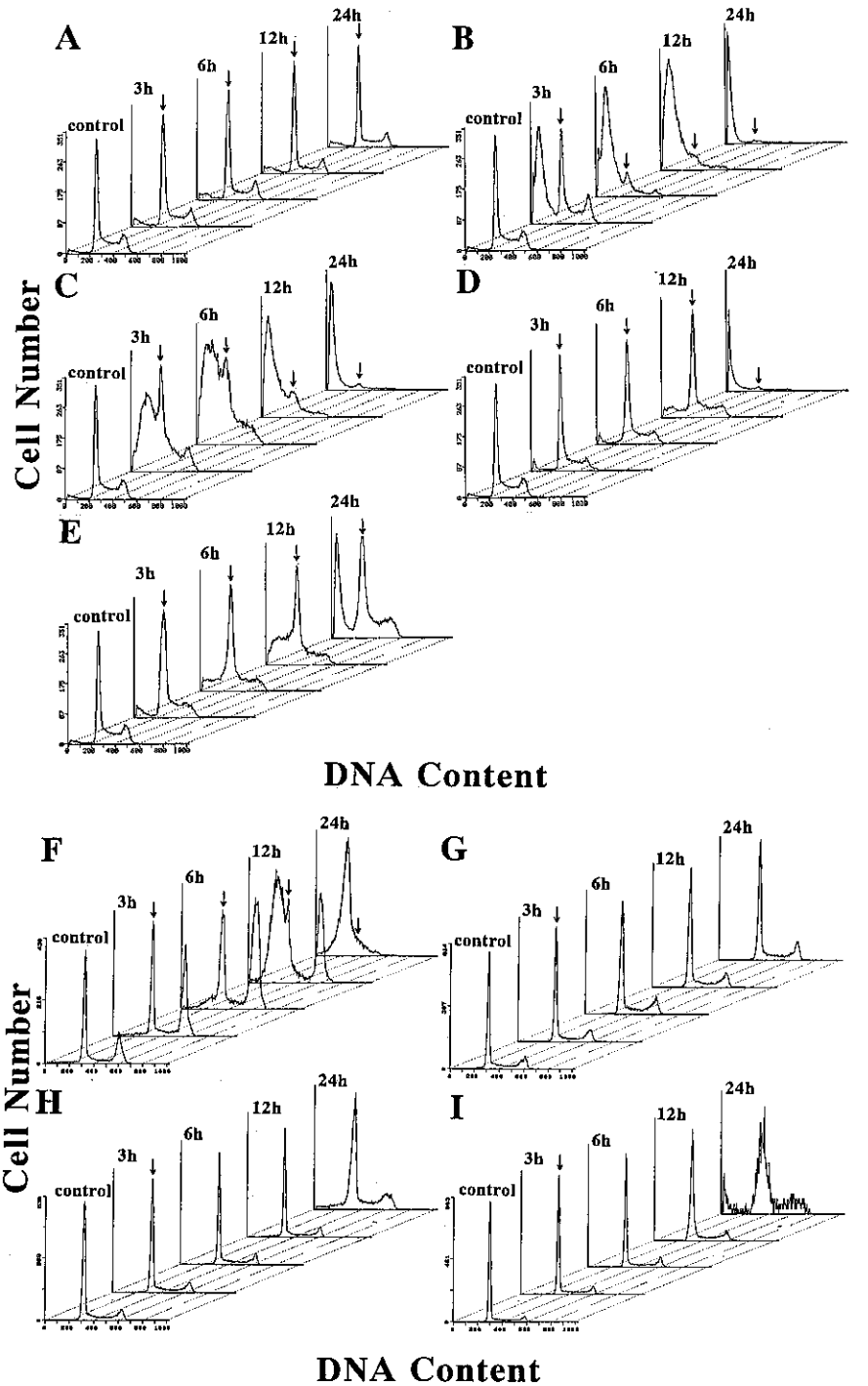


Fig. 1. Agarose gel electrophoresis of DNA from HL-60, HeLa S3 and WiDr cells treated with various doses of ethanol (for details, see "Materials and Methods"). The marker lane (M) contains DNA of 23,130, 9,416, 6,557, 4,361, 2,322, 2,027 and 564 base pairs. Con, control untreated cells. A, HL-60 cells: typical ladder patterns were evident at 3 to 24 h with 0.5 m*M* and at 3 to 6 h with 1.0 m*M* ethanol. With 0.2 m*M*, no change of DNA was seen. With 2.0 and 5.0 m*M*, only smear patterns were detected. B, Non-adherent HeLa S3 cells: a ladder pattern was observed only after 24 h with 0.5 m*M* ethanol. Control cells showed a slight smear pattern indicating some DNA degradation, probably due to the detachment. C, Adherent HeLa S3 cells: no significant DNA change was seen. D, Non-adherent WiDr cells: smear patterns were obvious with 0.5 m*M* ethanol. Control cells showed a slight smear pattern, like B.



pattern was seen and only smears were observed (Fig. 1A). DNA extracted from non-adherent HeLa S3 cells demonstrated a ladder pattern only with 0.5 m*M* ethanol after 24 h. The remainder showed no change of DNA (Fig. 1B). In the case of adherent HeLa S3 cells, no change of DNA was observed at any time or with any dose of ethanol (Fig. 1C). DNA extracted from non-adherent WiDr cells showed a smear pattern after exposure to 0.5 m*M* ethanol (Fig. 1D). Almost all WiDr cells were detached after treatment with more than 0.5 m*M*

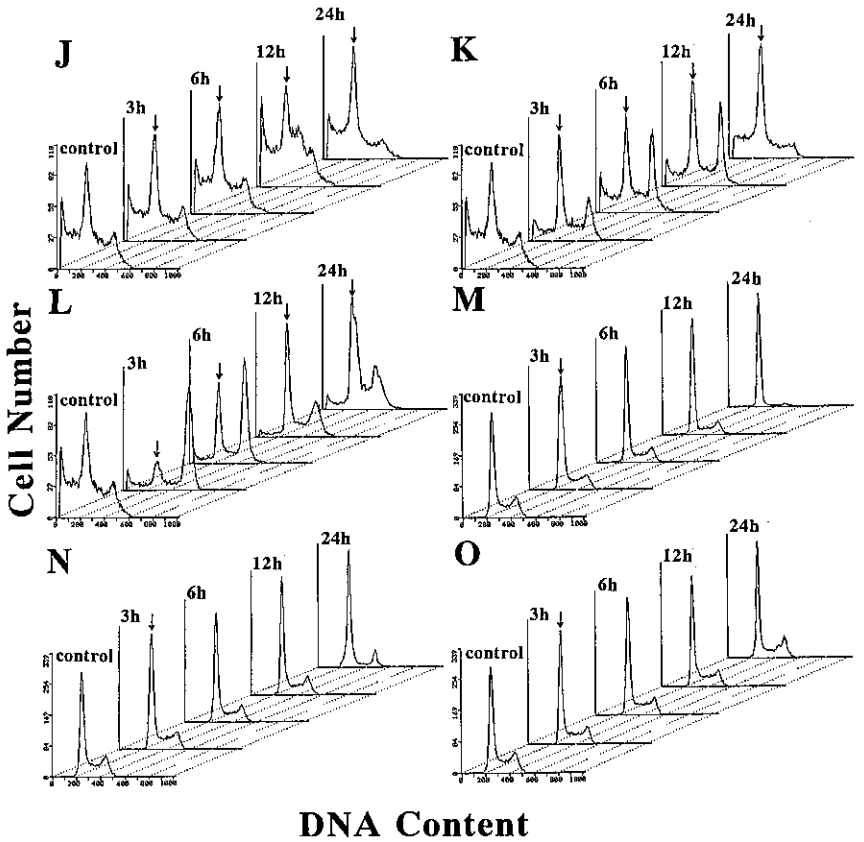


Fig. 2. DNA distribution histograms for HL-60, HeLa S3 and WiDr cells treated with ethanol (for details, see "Materials and Methods"). Arrows indicate G_0+G_1 cells. A–E, HL-60 cells (A, 0.2 m*M*; B, 0.5 m*M*; C, 1.0 m*M*; D, 2.0 m*M*; E, 5.0 m*M*). Peaks of A_0 cells (lower DNA content than G_0+G_1 cells) were apparent with 0.5 and 1.0 m*M* ethanol after 3 h treatment, and almost all the cells contained smaller amounts of DNA than diploid cells at 24 h. At higher doses (2.0 and 5.0 m*M*), A_0 cells appeared later than at lower doses. F–I, HeLa S3 cells (F, non-adherent with 0.5 m*M* ethanol; G, non-adherent with 1.0 m*M*; H, adherent with 0.5 m*M*; I, adherent with 1.0 m*M*). At 0.5 m*M*, elevation of the G_2+M peak followed by the appearance of A_0 cells (12 h) was seen in non-adherent cells, although no significant change was observed in the remainder. J–O, WiDr cells (J, non-adherent with 0.2 m*M* ethanol; K, non-adherent with 0.5 m*M*; L, non-adherent with 1.0 m*M*; M, adherent with 0.2 m*M*; N, adherent with 0.5 m*M*; O, adherent with 1.0 m*M*). With non-adherent WiDr cells, marked G_2+M peak elevation was noted at 0.5 and 1.0 m*M* ethanol, although A_0 cells were less prominent than with HL-60 or HeLa S3 cells. With 0.2 m*M*, slight accumulation in the S-phase was seen. In the adherent case, decrease of cells in the S-phase was observed with lower doses of ethanol (0.2 and 0.5 m*M*), although no significant change in the histograms was seen at 1.0 m*M*.

ethanol, and hence an examination of adherent WiDr cells could not be performed.

Changes in DNA histograms In HL-60 cells, typical peaks of A_0 cells were observed at doses of more than 0.5 m*M* ethanol (Fig. 2B). With 0.5 and 1.0 m*M* ethanol, A_0 cells were recognized as early as 3 h after beginning the exposure. In contrast, it was only after 24 h that A_0 cells were observed at 2.0 and 5.0 m*M* ethanol. With adherent HeLa S3 cells, no significant change was observed until the 24 h time point at 0.5 or 1.0 m*M* ethanol. Non-

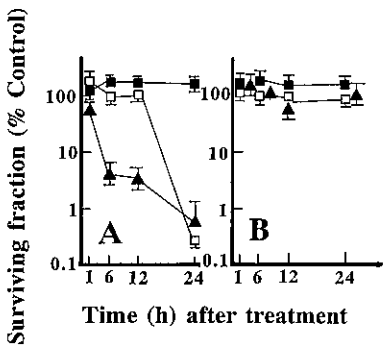


Fig. 3. Surviving fraction of HeLa S3 cells treated with ethanol (■, 0.2 m*M*; □, 0.5 m*M*; ▲, 1.0 m*M*). A, In non-adherent HeLa S3 cells, plating efficiency decreased after 12 h at 0.5 m*M* and after 1 h at 1.0 m*M*. B, With adherent HeLa S3 cells, little decrease of plating efficiency was seen ($n=10$; bars, SE).

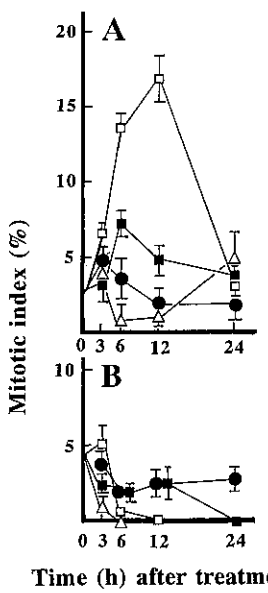


Fig. 4. Changes of mitotic indices of non-adherent HeLa S3 and HL-60 cells treated with ethanol (●, control; ■, 0.2 m*M*; □, 0.5 m*M*; △, 1.0 m*M*). A, The mitotic index of non-adherent HeLa S3 cells clearly increased after treatment with 0.5 m*M* ethanol, but not 0.2 or 1.0 m*M*. B, In HL-60 cells, mitotic indices were gradually decreased after treatment ($n=5$; bars, SE).

adherent HeLa S3 cells showed an elevation of the G_2+M peak after 3 h exposure to 0.5 m*M* ethanol, followed by the appearance of A_0 cells at 12 and 24 h, although no significant change was evident at 1.0 m*M* ethanol (Fig. 2G). With adherent WiDr cells, a decrease of S-phase cells was observed at low doses of ethanol (0.2 and 0.5 m*M*), although no significant change was seen at 1.0 m*M*. With non-adherent WiDr cells, however, marked elevation of the G_2+M peak was noted at 0.5 and 1.0 m*M* ethanol, although A_0 cells were most apparent at 0.2 m*M*, accompanied by an accumulation of cells in the S-phase (Fig. 2J).

Surviving fraction and mitotic indices With non-adherent HeLa S3 cells, the surviving fraction was decreased significantly after 12 h at 0.5 m*M* and after 1 h at 1.0 m*M* ethanol (Fig. 3A). No marked decrease was evident with adherent HeLa S3 cells at doses from 0.2 to 1.0 m*M* and exposures from one to 24 h (Fig. 3B). The mitotic index greatly increased at 0.5 m*M* in non-adherent HeLa S3 cells (Fig. 4A). In contrast, no M-phase cells were observed at any dose or time point for adherent HeLa S3 cells (data not shown). In the case of non-adherent WiDr cells, mitotic indices could not be calculated because pronounced morphological changes occurred in nuclei. No significant change in the mitotic index was seen for HL-60 cells (Fig. 4B). Morphological changes of HL-60 and non-adherent HeLa S3 cells are shown in Fig. 5.

DISCUSSION

Since the DNA ladder pattern on gel electrophoresis was reported,[10] the hypothesis that internucleosomal (linker) DNA is cut by activated endonuclease on apoptotic cell death has become generally accepted. In our study, however, the fact that a smear pattern appeared instead of a ladder pattern after 12 h treatment with ethanol (0.5 m*M*) in HL-60 cells may indicate that cutting of DNA in nucleosomes occurred during prolonged cytotoxic stimulation, and that linker DNA scission was not an endpoint. To demonstrate the presence of a DNA ladder, the procedures of extraction and gel electrophoresis are rather cumbersome and time-consuming, so that some simpler method for detection of oligonucleosomal fragmentation has been sought.

Various cell features have been found useful to characterize the mode of cell death,[11] i.e., activation of an endonuclease in apoptotic cells resulting in loss of low-molecular-weight DNA through permeabilization of the membrane leads to decreased stainability with DNA-specific fluorochromes such as propidium iodide. Therefore, the appearance of cells with a lower DNA stainability than that of G_0+G_1 cells (so-called A_0 cells or sub-G_1 peak) has been suggested as a marker of cell death by apoptosis.[2,14] Cell death by necrosis, on the other hand, due to poisoning or ischemia, is characterized by swelling of mitochondria and immediate loss of plasma membrane integrity.[4] At a certain phase of necrotic cell death, disappearance of chromatin and random degrada-

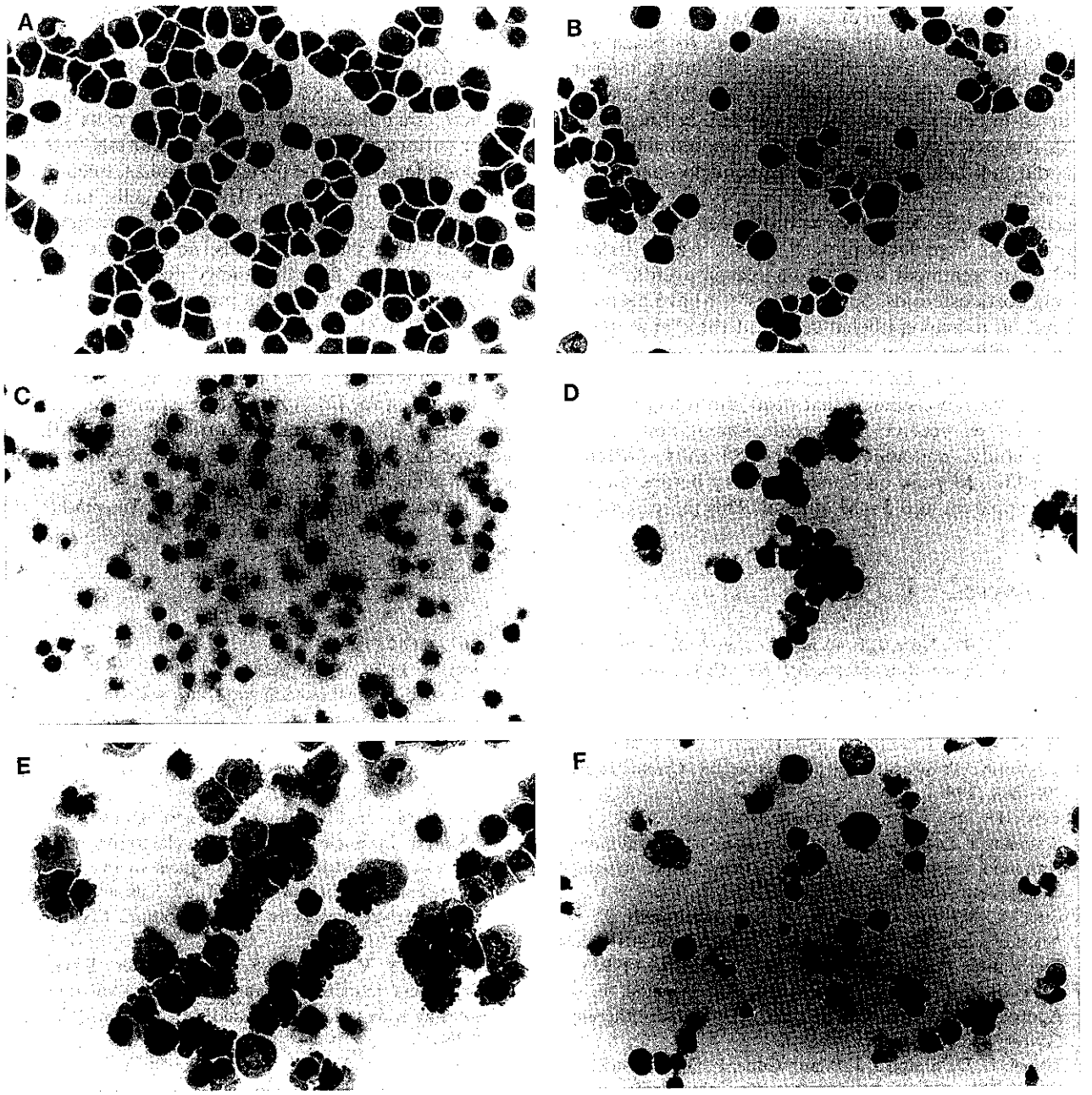


Fig. 5. Light microscopic morphology of HL-60 and HeLa S3 cells treated with ethanol (May-Giemsa, ×320). A–C, HL-60 cells (A, control; B, 0.5 m*M* at 3 h; C, 2.0 m*M* at 3 h). Shrinkage of cytoplasm and nuclear fragmentation were seen at 0.5 m*M*, but not at 2.0 m*M*. D–F, non-adherent HeLa S3 cells (D, control; E, 0.5 m*M* at 6 h; F, 0.5 m*M* at 24 h). Bulging of cytoplasm and many M-phase cells were seen at 6 h, followed by nuclear fragmentation at 24 h, but to a lesser extent than in the case of HL-60.

tion of DNA occur, which may result in loss of DNA content.[4,5]

Lennon *et al.*[12] reported that 5% (=0.86 m*M*) ethanol caused apoptotic cell death of HL-60 cells, whereas 10% caused necrotic cell death. In our study, peaks of A_0 cells of HL-60 were recognized early (3 h) with low doses (0.2 and 0.5 m*M*) of ethanol, but later (24 h) at higher doses (2.0 and 5.0 m*M*). DNA gel electrophoresis

revealed a ladder pattern at 0.5 and 1.0 m*M*, but not 2.0 and 5.0 m*M*, so that the appearance of A_0 peaks with these latter doses cannot be considered due to internucleosomal DNA breaks. We conclude that DNA loss also occurred in cells that underwent necrotic cell death and DNA loss after necrotic cell death required more time than did cell death after DNA oligonucleosomal fragmentation.

Apoptotic cell death has mainly been studied with thymocytes[13–15] or leukemic cell lines[16–18] after irradiation[19–21] or treatment with antineoplastic agents[22–24] including corticosteroids. Some studies[25,26] have been carried out on human epithelial cells, but these have generally dealt with adherent cells. Many human epithelial cell lines grow as monolayers attached to the culture flasks, and it has been considered that dead and dying cells are detached after receiving cytotoxic stimulation. In the present study, non-adherent HeLa S3 cells showed a marked G_2+M peak on flow cytometry before A_0 cells became apparent at 0.5 m*M* ethanol. This increase of the G_2+M population was attributed to an accumulation of cells in the M-phase on counting mitotic figures. With regard to the cell reproductivity, the surviving fraction of non-adherent HeLa S3 cells significantly decreased with 0.5 m*M* ethanol after 24 h, when these cells showed a ladder pattern of DNA in gel electrophoresis. It is therefore considered that the period of M-phase accumulation was partially reversible for HeLa S3 cells, but became irreversible after DNA oligonucleosomal fragmentation occurred. With the HL-60 cells, no M-phase accumulation was seen by flow cytometry and a ladder pattern could be detected by gel electrophoresis of DNA after 3 h treatment with ethanol, although 24 h exposure was necessary for non-adherent HeLa S3 cells. We consider that this difference in the presence of M-phase arrest and the time taken for DNA oligonucleosomal fragmentation between HL-60 and HeLa S3 probably reflects the time required for expression of oncogenes and/or tumor suppressor genes.

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