Potent Inhibition of Cell Density-Dependent Apoptosis and Enhancement of Survival by Dimethyl Sulfoxide in Human Myeloblastic HL-60 Cells

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Human myeloblastic cell line HL-60 cells undergo apoptosis during in vitro culture in a cell density-dependent manner, and this cell density-dependent apoptosis was observed when the concentration of cultured cells exceeded 8–10 $\times$ 10^5 cells/ml. Dimethyl sulfoxide (DMSO), a differentiation inducer of HL-60 cells, did not amplify, but rather potently inhibited, this apoptosis. In a low density culture condition, DMSO attenuated proliferation of HL-60 cells in spite of its inhibition of apoptosis. In contrast, DMSO did support cell survival under high cell density conditions, and DMSO-treated HL-60 cells reached an extremely high concentration of 2–3 $\times$ 10^6 cells/ml, a condition which could never be possible in a usual culture environment. Thus, DMSO exerted dual effects on cell proliferation, i.e., growth inhibition and apoptosis inhibition, and the sum of these effects resulted in an apparently distinct phenomenon according to the culture conditions including cell density. **J. Cell. Physiol. 174:135–143, 1998.**

Apoptosis is a way of cell death that is programmed genetically and occurs actively. It has been described as "shrinkage necrosis" or "coagulative necrosis" by Kerr et al. (1972). Detailed analyses have been performed since then and its critical and important roles in a wide variety of biological systems have been determined (Hale et al., 1996). In the process of apoptosis, the cells show morphological changes including nuclear condensation, extensive plasma membrane ruffling, and formation of "apoptotic bodies," which are small, roughly spherical or ovoid cytoplasmic fragments that contain pyknotic remnants of nuclei (Kerr et al., 1972). The biochemical change is the formation of DNA fragments by endonucleases, and is detected as a characteristic DNA ladder by agarose gel electrophoresis (Hale et al., 1996).

Both dimethyl sulfoxide (DMSO) and all-trans retinoic acid (ATRA) are well-known inducers of differentiation of myeloid cells in an in vitro culture system, and it has been repeatedly reported that both agents induce granulocytic differentiation of human myeloblastic cell line HL-60 (Collins et al., 1978; Kitagawa et al., 1984; Matsushima et al., 1992). During the in vitro differentiation process, both agents usually induce growth inhibition of the cells. In addition, differentiated cells, which have lost their ability to proliferate, are considered to terminate their life by apoptosis (Martin et al., 1990).

These in vitro phenomena, i.e., growth inhibition, differentiation, and apoptosis, may also occur in vivo in this sequence during human granulocytic cell production, which might, in turn, contribute to homeostasis of normal hematopoiesis of the granulocytic series. Successful treatment of acute promyelocytic leukemia by ATRA (Smith et al., 1992) clearly demonstrated that these sequential phenomena really occur in vivo during the differentiation induction therapy of abnormal and malignant leukocytes.

In addition to this kind differentiation-associated apoptosis, several different modes of apoptosis appear to exist in human myeloid cells. For example, exogenous stimuli such as tumor necrosis factor (TNF) potently induce apoptosis in normal human neutrophils

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(Takeda et al., 1993), and its intracellular mechanism including the involvement of an interleukin-1 β converting enzyme (ICE) pathway has recently been reported (Hale et al., 1996). Exponentially growing HL-60 cells undergo apoptosis in their minor population, as determined by sensitive assay systems (Ikeda-Nishizawa et al., 1996). The latter apoptosis may be called "spontaneous" apoptosis, although its mechanism is still largely unknown.

In the present study, we unexpectedly found that DMSO but not ATRA inhibited "spontaneous" apoptosis of HL-60 cells, and we characterized this unique effect of this polar compound. We also clarified that this spontaneous apoptosis depends on the cell density of the culture system, and found that the inhibitory effect of DMSO on apoptosis was observed only when DMSO was added to the culture before the critical point of cell density. Interestingly, DMSO, which usually inhibits proliferation of HL-60 cells, apparently potentiated cell growth under high density culture condition by markedly inhibiting apoptosis. These dual effects of DMSO on the proliferation of HL-60 cells show unique aspects of this differentiation-inducing agent, which are completely different from those of other differentiation-inducing agents including ATRA.

MATERIALS AND METHODS

Reagents

Cytochrome C, superoxide dismutase, phorbol myristate acetate (PMA), and ATRA were purchased from Sigma Chemical Co., St. Louis, MO; DMSO, RNase A, and ethidium bromide were from Wako Pure Chemicals, Tokyo, Japan; RPMI 1640 medium was from Gibco Laboratories, Grand Island, NY; fetal calf serum (FCS) from JRH Bioscience, Lenexa, KS; Conray from Mallinckrodt Inc., St. Louis, MO; Ficoll from Pharmacia Fine Chemicals Inc., Piscataway, NJ; Proteinase K from Boehringer Mannheim, Mannheim, Germany; nitrocellulose membrane from Bio-Rad, Richmond, CA; monoclonal anti-human Fas antibody from MBL, Nagoya, Japan; monoclonal anti-human Bcl-2 antibody from Pharmingen, San Diego, CA; polyclonal anti-human c-Myc antibody from UBI, Lake Placid, NY; polyclonal anti-human Mcl-1 antibodies from Santa Cruz Biotechnology Inc., Santa Cruz, CA; and alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgG antibodies were from Promega, Madison, WI. Other reagents for genomic DNA analysis and Western blotting were purchased from Sigma Chemical Co., St. Louis, MO.

Culture and preparation of cells

HL-60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml), as described (Kitagawa et al., 1984). For induction of differentiation, HL-60 cells were seeded at 4×10^4 cells/ml and grown in the presence or absence of 1 μM ATRA and 1% DMSO for 1–5 days. Cultured cells were harvested after 1–5 days of cultivation with inducers of differentiation, washed three times with phosphate-buffered saline (PBS), and then suspended in PBS or Hanks' balanced salt solution (HBSS).

Determination of superoxide release

Superoxide (O_2^-) was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome C, and the continuous assay was performed in a Hitachi 556 spectrophotometer (a double-wavelength spectrophotometer; Hitachi Ltd., Tokyo, Japan), equipped with thermostatted cuvette holder (37°C) as described (Yuo et al., 1993). Final cell concentration was 1×10^6 cells/ml. The reduction of cytochrome C was measured at 550 nm with a reference wavelength at 540 nm, and the time course of cytochrome C reduction (absorbance change at 550−540 nm) was followed on the recorder. The amount of O_2^- release was calculated from the linear portion of the cytochrome C reduction for PMA.

Genomic DNA analysis

The detection of DNA fragments was performed according to the method by Herrmann et al. (1994) with minor modifications. 3×10 cells were suspended in 50 μl lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5), and lysed for 10 sec. After centrifugation for 5 min at 1,600 g, the supernatant was collected. Another 50 μl of lysis buffer was added to the pellets and the extraction procedure was repeated. After sodium dodecyl sulfate (SDS; final concentration 1%) was added to the total 100 μl of supernatant, these solubilized samples were treated with RNase A (final concentration 5 μg/ml) for 2 hours at 56°C followed by digestion with Proteinase K (final concentration 2.5 μg/ml) for 2 hours at 56°C. After addition of 1/2 volume of 10 M ammonium acetate, the DNA was precipitated with 2.5 volumes of ethanol. Precipitated DNA was dissolved in 10 μl TE (Tris-EDTA buffer) and mixed with 2 μl of 6× loading buffer (0.25% bromophenol blue (BPB), 30% glycerol). Horizontal electrophoresis was performed with 2% agarose gel and 0.5× TBE buffer (Tris-borate/EDTA electrophoresis buffer; 45 mM Tris-borate, 1 mM EDTA) at 100 V, using a mini electrophoretic system, MUPID-2 (Cosmo Bio, Tokyo, Japan), with 500 ng/ml ethidium bromide for visualization of DNA. The ϕ X174/Hae III digest was used as a molecular marker.

Morphological evaluation of cells

Light microscopic examination of cell viability and morphology was done to determine the apoptosis and differentiation of HL-60 cells. For the determination of cell viability, a cell count was made on the hemocytometer using the trypan blue dye exclusion test. For the morphological evaluation of apoptosis and differentiation, cells suspended in HBSS were caused to adhere to slide glasses by centrifugation using Cytospin 2 (Shandon Inc., Pittsburgh, PA) and cytospin slides were then subjected to Wright-Giemsa staining for light microscopic examination of the cells.

Western blotting

Cells were suspended in an ice-cold solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% deoxychorate. The cell suspension was mixed 1:1 with 2× sample buffer (4.6% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.004% BPB in 83 mM Tris-HCl, pH 6.8), heated at 100°C for 5 min, and then loaded onto 7.5% or 12.5%

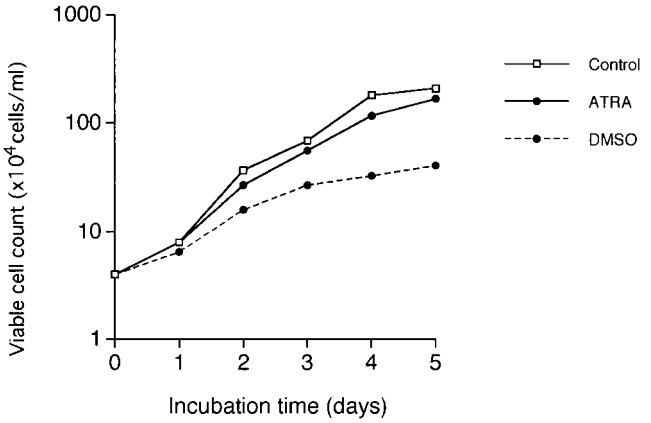

Fig. 1. Growth curves of HL-60 cells. HL-60 cells were diluted to the concentration of 4×10^4 cells/ml on day 0, and were then incubated with or without DMSO (1%) or ATRA (1 μM) for 1 to 5 days. The cells were harvested every 24 hours and a viable cell count was performed using the trypan blue dye exclusion method. During 5 days of culture, no dead cells determined by this method were observed.

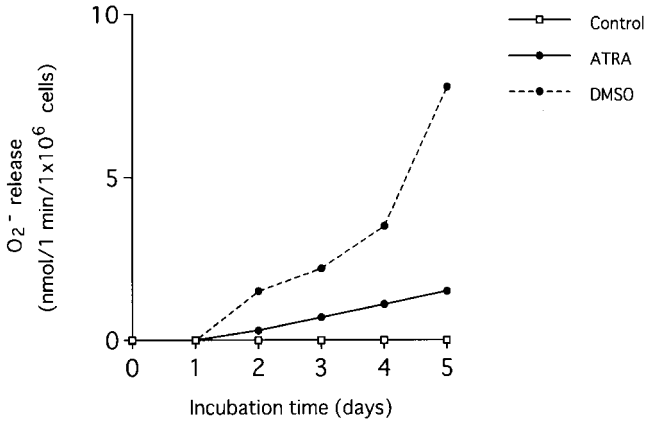

Fig. 2. Superoxide-producing capacity of HL-60 cells. HL-60 cells were diluted to the concentration of 4×10^4 cells/ml on day 0, and were then incubated with or without DMSO (1%) or ATRA (1 μM) for 1 to 5 days. The cells were harvested every 24 hours and superoxide (O_2^-) release stimulated by PMA (100 ng/ml) was determined by the reduction of cytochrome C.

sodium dodecyl sulfate gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 2 mA/cm^2 for 2 hr at 4°C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffer (150 mM Tris-HCl, pH 7.6) containing 3% bovine serum albumin (BSA) for 1 hr at 25°C. The blots were then washed in PBS containing 0.05% Tween 20 (PBST), and incubated with antibodies raised against four intracellular apoptosis-related proteins in Tris-buffer containing 1% BSA for 4 hr at 25°C. The primary antibody was removed and the blots were washed three times in PBST. To detect antibody reactions, the blots were incubated for 2 hr with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG antibody diluted 1:2,000 in Tris-buffer containing 1% BSA, washed three times with PBST, and then placed in a buffer containing 100 mM Tris-HCl (pH 9.7), 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 200 μg/ml nitroblue tetrazolium, and 200 μg/ml 5-bromo-4-chloro-3-indolylphosphate for 10–30 min at 25°C. Enzymatic color development was stopped by rinsing the membranes in deionized water.

RESULTS

Proliferation and apoptosis during differentiation of human myeloblastic HL-60 cells

Figure 1 shows a growth profile of human myeloblastic cell line HL-60 during differentiation induction by DMSO and ATRA. As previously reported (Collins et al., 1978; Kitagawa et al., 1984; Matsushima et al., 1992), both agents induced functional differentiation of HL-60 cells as determined by superoxide-producing capacity, and the differentiation-inducing effect of DMSO on our clone of HL-60 cells was more potent than that of ATRA (Fig. 2). As shown in Figure 1, DMSO suppressed proliferation speed of the exponentially growing phase of HL-60 cells seeded at low concentration (4×10^4 cells/ml) and this suppressive effect was more potent than that of ATRA. During these processes of proliferation and/or differentiation, HL-60 cells underwent apoptosis as determined by DNA fragmentation assay even in the control culture (Fig. 3, left panel). This kind of apoptosis was formerly called "spontaneous" apoptosis of this cell line in its minor population (less than 5%), and was hardly detectable by the conventional method (trypan blue dye exclusion method) used to detect dead cells (see legend for Fig. 1). ATRA-induced cells also show DNA fragmentation to some extent. Interestingly, DMSO potently inhibited the "spontaneous" apoptosis of HL-60 cells in a time-dependent manner (Fig. 3).

Spontaneous apoptosis observed in the control culture was apparently dependent on the culture periods except day 0 (Fig. 3). One possible explanation of this finding is that observed apoptosis depends upon cell density from days 1 to 5 of cultivation, and relatively prominent apoptosis at day 0 may reflect the cell density just before the start of the study. To obtain information on this possibility, we compared the extent of DNA fragmentation of HL-60 cells at three points of cell density during the proliferation. Figure 4 clearly indicates the cell concentration-dependent DNA ladder formation of HL-60 cells during in vitro culture system. The cell density-dependent mechanism of apoptosis observed in HL-60 cells was characterized and analyzed in detail elsewhere and established as one of the important cell growth regulatory systems of this cell line (Saeki et al., 1997). Thus, DMSO was found to be an agent inhibiting the cell density-dependent apoptosis of HL-60 cells.

Potent inhibition of cell density-dependent apoptosis and enhancement of cell growth by DMSO

Data from Figures 1 and 3 show diverse effects of DMSO on the biology of HL-60 cells, i.e., inhibition of

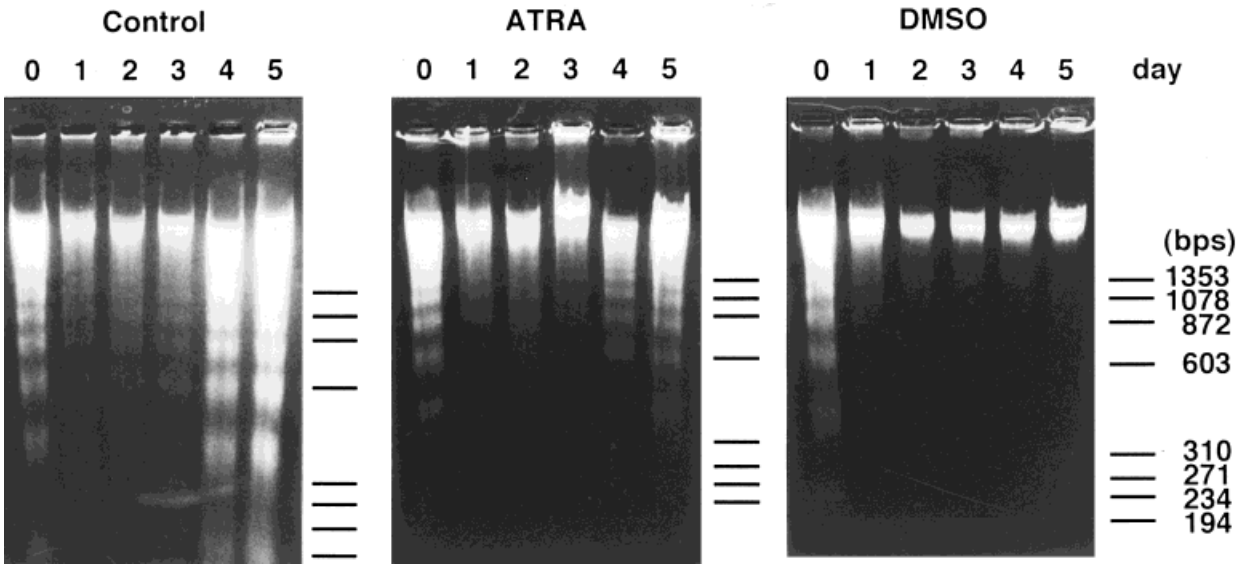

Fig. 3. DNA fragmentation of HL-60 cells. Exponentially growing HL-60 cells were diluted to the concentration of 4×10^4 cells/ml on day 0, and were then incubated with or without DMSO (1%) or ATRA (1 μM) for 1 to 5 days. The cells were harvested every 24 hours and DNA fragmentation analysis was performed as described in Materials and Methods. The molecular markers are shown to the right of the figures.

proliferation (Fig. 1) and inhibition of apoptosis (Fig. 3). Although these dual effects of DMSO are of interest, there are two concerns regarding these findings. First, it is not clear whether the observed inhibition of DNA ladder formation (Fig. 3) really leads to a net enhancement of cell survival, since DMSO at the same time has a suppressive effect on proliferation of this cell line (Fig. 1). Second, we cannot rule out the possibility that the observed inhibition of apoptosis by DMSO (Fig. 3) results simply from the inhibitory effect of this agent on HL-60 cell proliferation, since "spontaneous" apoptosis of HL-60 cells was strictly dependent on the cell density (Fig. 4), and DMSO-treated HL-60 cells remained in relatively low cell density conditions (Fig. 1).

To rule out these possibilities and further characterize the inhibitory effects of DMSO on cell density-dependent apoptosis, we investigated the effects of this substance on cells cultured in intermediate to high cell concentrations. When the cultivation of HL-60 cells with 1% DMSO was started at relatively high cell concentration (1×10^6 cells/ml), at which the DNA ladder formation of the cells was thought to have already begun (Fig. 4), DMSO did not completely inhibit or reverse fragmentation of DNA and substantial DNA ladder formation was observed in both DMSO(−) and DMSO(+) cultures (Fig. 5, left panel). In these situations, however, HL-60 cells reached an extraordinarily high concentration (2.6×10^6 cells/ml) only in the presence of DMSO (Fig. 5, left panel), clearly showing the potent inhibition of apoptosis and enhancement of survival by DMSO.

When the cultivation of HL-60 cells with DMSO was started at the cell concentration of $5–8 \times 10^5$ cells/ml, at which no or only a marginal level of DNA ladder formation was observed (data not shown), DMSO completely inhibited the DNA ladder formation of HL-60 cells during 24 hours (data not shown) and 48 hours (Fig. 5, right panel) of cultivation. Also in these DMSO(+) cultures, HL-60 cells reached an extremely high cell concentration particularly at 48 hours of cultivation ($2.0–2.3 \times 10^6$ cells/ml). These findings indicate not only potent inhibition of apoptosis by DMSO but also resultant growth advantage of the cells in DMSO(+) cultures, at least in cultures with relatively high cell concentrations.

To confirm the potent inhibition of apoptosis by DMSO in high concentrated cultures and determine the growth advantage of cells under these conditions, we monitored growth curves of viable HL-60 cells by the trypan blue dye exclusion method (Fig. 6). When HL-60 cells were cultured without DMSO in a high cell concentration, growth of the cells was saturated after 2 days of culture at the concentration of approximately 2×10^6 cells/ml (Fig. 6). Growth-saturated HL-60 cells could no longer proliferate, and the viable cell number decreased thereafter with concomitant increase of dead cells. DMSO-treated HL-60 cells, on the other hand, continued to proliferate and reached an extremely high concentration (more than 2.5×10^6 cells/ml). These cells survived with a minimal number of dead cells under a high cell density condition even at 5 days of culture, when more than half of the HL-60 cells died without DMSO.

Morphological examination of HL-60 cells cultured in the presence of DMSO showed rapid cell differentiation, i.e., lobulated nuclei with concomitant reduction of cell size even at 1 day of culture with DMSO (Fig. 7), while in control cultures we observed many dead cells with typical morphology of apoptosis (apoptotic body) among living cells with immature morphology

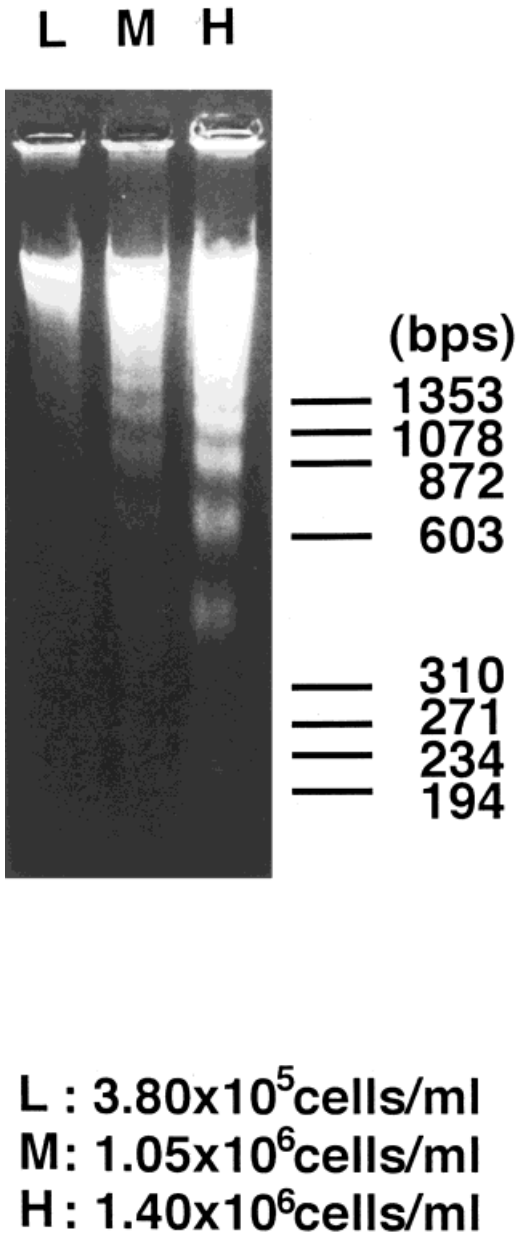

Fig. 4. Cell density-dependent DNA ladder formation of HL-60 cells. HL-60 cells were grown without any inducers of differentiation and were harvested at three points, when the cell density was low (L), medium (M), or high (H). Actual cell density is indicated in the lower portion of the figure. DNA fragmentation analysis was performed as described in Materials and Methods, and the molecular marker is shown to the right in the figure.

(Fig. 7). These findings demonstrated both potent induction of differentiation and potent inhibition of apoptosis in HL-60 cells cultured with DMSO.

Western blot analysis of apoptosis-related proteins in HL-60 cells

To obtain information on the molecular basis for regulation of apoptosis during differentiation induction and high cell density culture, we determined the protein content of apoptosis-related intracellular molecules using Western blotting for each protein. We chose four well-known intracellular apoptosis-related molecules: Fas, Bcl-2, Mcl-1, and c-Myc (Hale et al., 1996), and four culture conditions: low cell density culture without DNA ladder formation, high cell density culture with DNA ladder, ATRA-induced cells with DNA ladder, and DMSO-induced cells without DNA ladder.

As shown in Figure 8, intracellular content of Fas was not changed irrespective of which culture condition was selected. The content of Bcl-2 and c-Myc, however, was markedly decreased in differentiated HL-60 cells, and the intracellular amount of c-Myc oncoprotein was also decreased under high cell density culture conditions. Interestingly, the intracellular level of Mcl-1, which was originally found as a differentiation-related molecule (Kozopas et al., 1993), was not altered during differentiation induction by either ATRA or DMSO, but, rather, decreased in high cell density cultures with DNA ladder formation. These findings may suggest some relationship between decrement of Mcl-1 and c-Myc protein and cell density-dependent apoptosis. However, no DMSO-specific change of any protein content, which might explain DMSO-specific inhibition of apoptosis, was observed.

DISCUSSION

There have been recent reports of several experimental systems showing cell density-dependent apoptosis in cultured cells. Most of these studies, however, showed apoptosis observed under low density rather than high density culture conditions (Falk et al., 1993; Ishizaki et al., 1994,1995; Mathieu et al., 1995; Huss et al., 1995). In these systems of cell culture, contact with other cells or high cell density could protect cells from apoptosis, and the mechanism of this kind of apoptosis inhibition has been thought to be due to certain survival signals provoked by direct cell-to-cell contact or soluble growth factors produced and released from the cells in an autocrine or paracrine manner. Thus, low cell density could induce apoptosis of the cells via lack or reduction of the survival signal mediated by these mechanisms.

High cell density may also induce apoptosis, and this type of apoptosis could have an important physiological role in vivo in several situations, such as prevention of tissue hyperplasia and maintenance of appropriate tissue volume (Bursch et al., 1984; Columbano et al., 1985). However, the mechanism of high cell density-dependent apoptosis is not well characterized and is still largely unknown. As is the case with low cell density apoptosis, cell-to-cell contact or soluble factor may represent one mechanism, though high cell density provokes an apoptotic signal rather than a survival signal in this case. According to our preliminary study, concentrated HL-60 cells seem to produce and release a certain unknown soluble low molecular weight peptide factor which induces apoptosis of their own, suggesting an autocrine or paracrine mechanism of cell density-dependent apoptosis in HL-60 cells (Saeki et al., 1997). At present, however, we cannot

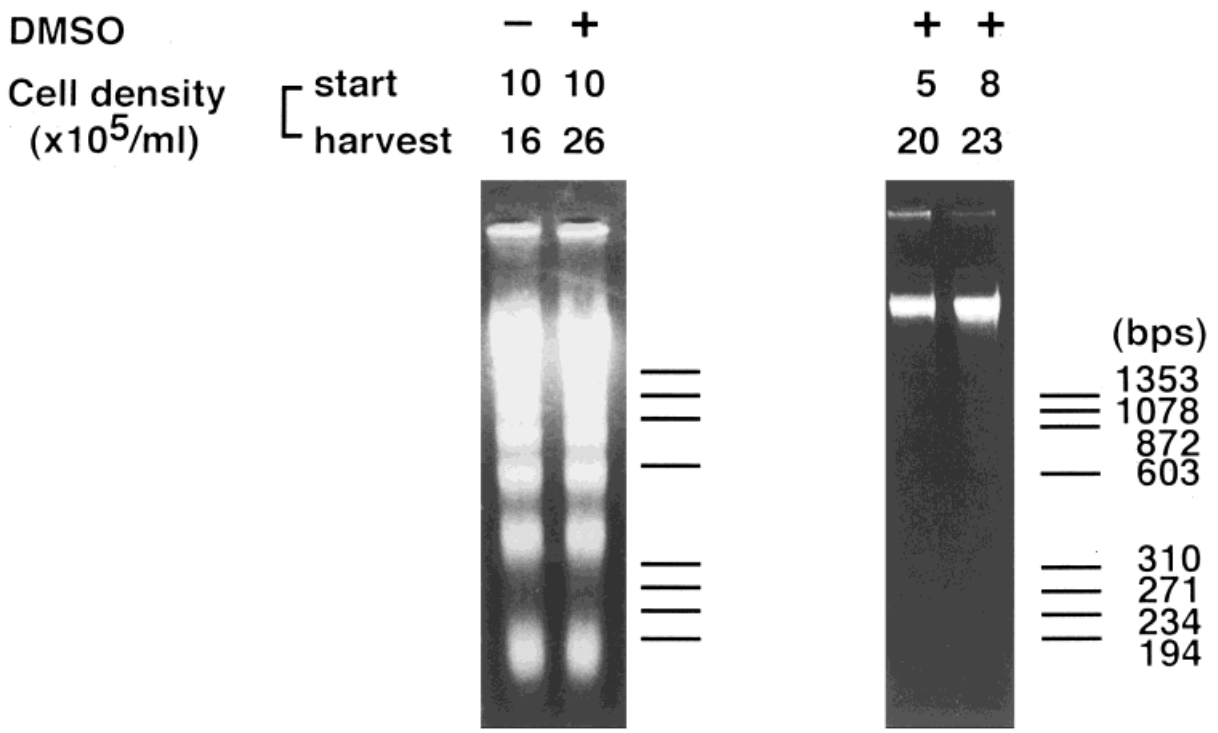

Fig. 5. DNA ladder formation of HL-60 cells cultured at intermediate to high cell density. HL-60 cells were cultured for 24 (left) or 48 (right) hours under intermediate to high cell density conditions with or without DMSO. Cell density at start and end of culture is indicated in the upper portion of the figure. DNA fragmentation analysis was performed as described in Materials and Methods, and the molecular markers are shown at the right of the figures.

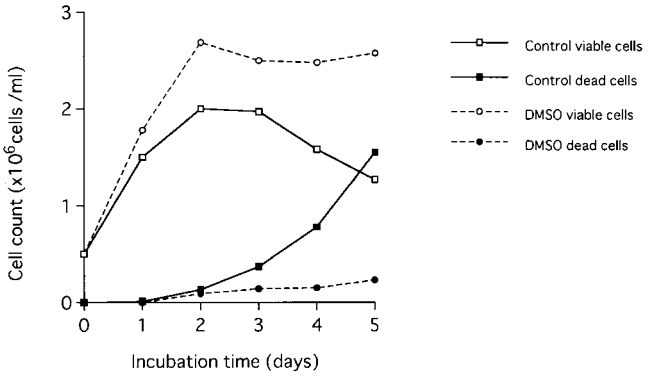

Fig. 6. Growth curves and viabilities of HL-60 cells cultured with or without DMSO at intermediate to high cell density. HL-60 cells were diluted to the concentration of 5×10^5 cells/ml on day 0, and were then cultured with or without DMSO (1%) for 1 to 5 days. The cells were harvested every 24 hours and the viable and dead cell count was performed using trypan blue dye exclusion method.

completely rule out the direct cell-to-cell interaction mechanism. Irrespective of the mechanism of high cell density apoptosis, we demonstrated potent inhibition of this kind of apoptosis by DMSO using an in vitro culture system of the suspended hematopoietic cell line, HL-60.

DMSO is a well-known differentiation inducer in human myeloid cells and its effect on cell proliferation is generally of a suppressive nature (Collins et al., 1978). This is also the case with HL-60 cells as shown in the present experiment (Fig. 1). The suppressive effect of DMSO on cell proliferation seems to reflect cell cycle arrest at the G1 phase (Horiguchi-Yamada and Yamada, 1993) or reduction of cell cycle speed. These findings might give the impression that myeloid differentiation of leukemic cell lines is closely associated with their growth inhibition, and that nonspecific inhibition of cell proliferation may lead to differentiation. On the other hand, we observed potentiation of cell survival and the resultant increase in cell number by DMSO in HL-60 cells at high cell density. This is a rare phenomenon in which enhancement of cell survival and induction of differentiation were simultaneously observed in transformed cell lines, though the simultaneous combination of differentiation and proliferation are frequently seen in normal hematopoietic cells stimulated by hematopoietic growth factors (Robinson and Quesenberry, 1990).

In the present study, we observed differentiation-associated decrease in the intracellular amount of c-Myc and Bcl-2 oncoproteins, a phenomenon which was consonant with the previous observations (Westin et al., 1982; Delia et al., 1992). Decrease in these two proteins was consistently observed in both ATRA- and DMSO-induced differentiation, sug-

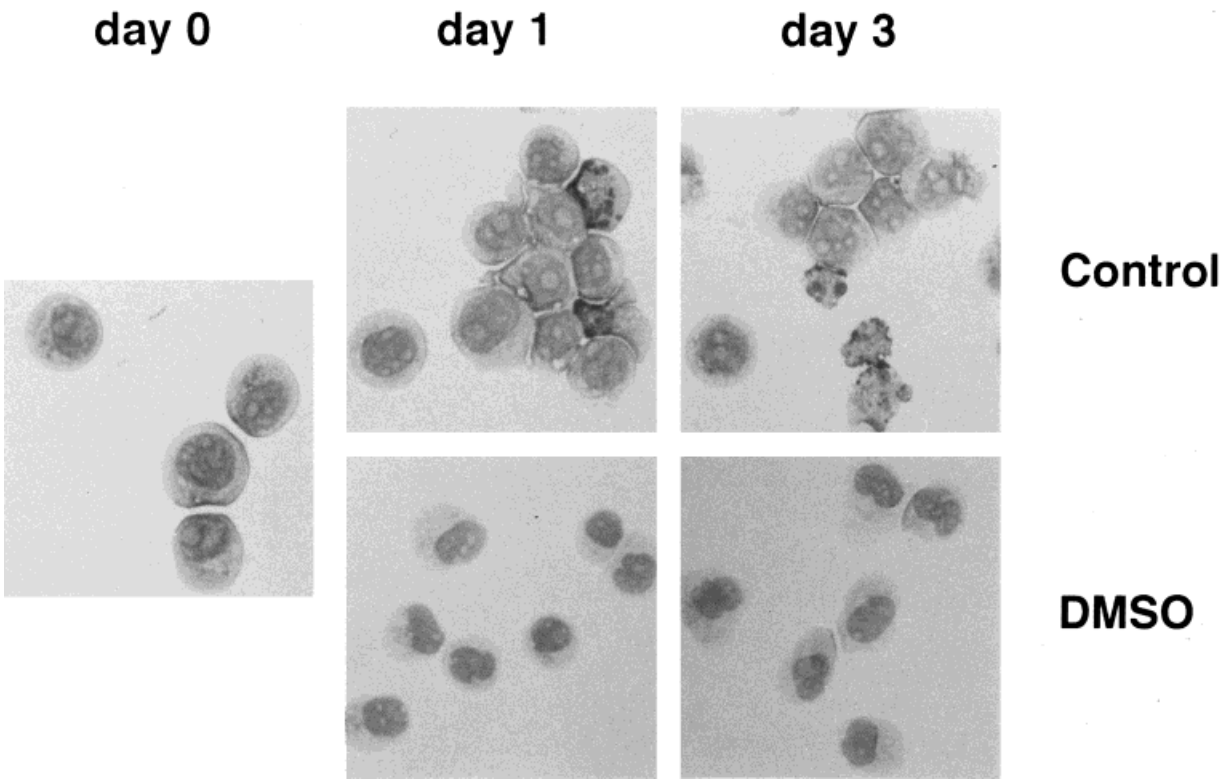

Fig. 7. Morphology of HL-60 cells cultured with or without DMSO at intermediate to high cell density. HL-60 cells were cultured under the same conditions as described in the legend for Figure 5, and cytospin specimens of the cells were prepared as described in Materials and Methods to evaluate the morphology.

gesting the DMSO-specific effect could not really be explained by the reduction of these oncoproteins. In regard to the cell density-dependent phenomenon, the intracellular level of Mcl-1 and c-Myc was decreased under high density culture conditions. The former protein, Mcl-1, was found to be one member of the Bcl-2 family and its possible role in cell biology was proposed to be myeloid differentiation rather than inhibition of apoptosis (Kozopas et al., 1993), suggesting unique characteristics of this protein within the family. Expression of Mcl-1, however, was not altered during differentiation induction by ATRA or DMSO in HL-60 cells. This indicates that, at least in differentiation of HL-60 cells, Mcl-1 plays only a minimal role, if any at all. On the other hand, substantial reduction of Mcl-1 and c-Myc at high cell density implies that these proteins function in proliferation, apoptosis, and/or cell cycle. Further study is required to answer these questions and hypotheses.

In addition to these intracellular apoptosis-related molecules, an autocrine inducer of apoptosis which plays an important part in the cell density-dependent apoptosis of HL-60 cells (Saeki et al., 1997) may also contribute to the mechanisms for DMSO inhibition of apoptosis. In fact, we found that conditioned medium of DMSO-treated HL-60 cells contained less activity to induce apoptosis of HL-60 cells than conditioned medium of HL-60 cells cultured without DMSO, suggesting decreased production of the apoptosis-inducer in the DMSO-treated HL-60 cells (unpublished observations). However, we could not rule out the apoptosis-inhibitory effect of DMSO remaining in the conditioned medium of DMSO-treated cells. These problems cannot be easily resolved until a specific quantification method of apoptosis inducing factor (other than bioassay) is established.

The present study clarified the differentiation-inducing and survival-enhancing effects of DMSO on the human myeloblastic cell line. The physiological significance of this phenomenon is not yet clear, partly because of the fact that DMSO is not a naturally occurring substance. In this context, it is of interest and importance that the physiological cytokine transforming growth factor (TGF) β 1 also potently inhibited cell density-dependent apoptosis of HL-60 cells (Saeki et al., 1997). In contrast to DMSO, TGF β 1 neither suppressed cell cycle speed of exponential growth nor induced morphological and functional differentiation of HL-60 cells. On the other hand, Maeda et al. (1993) have reported suppression of high cell density-dependent apoptosis in cultured hepatocytes by heparin. Previous studies from our own and other laboratories, together with the current investigation, might provide some clues to physiological regulation of the cell density-dependent phenomena of prolifera-

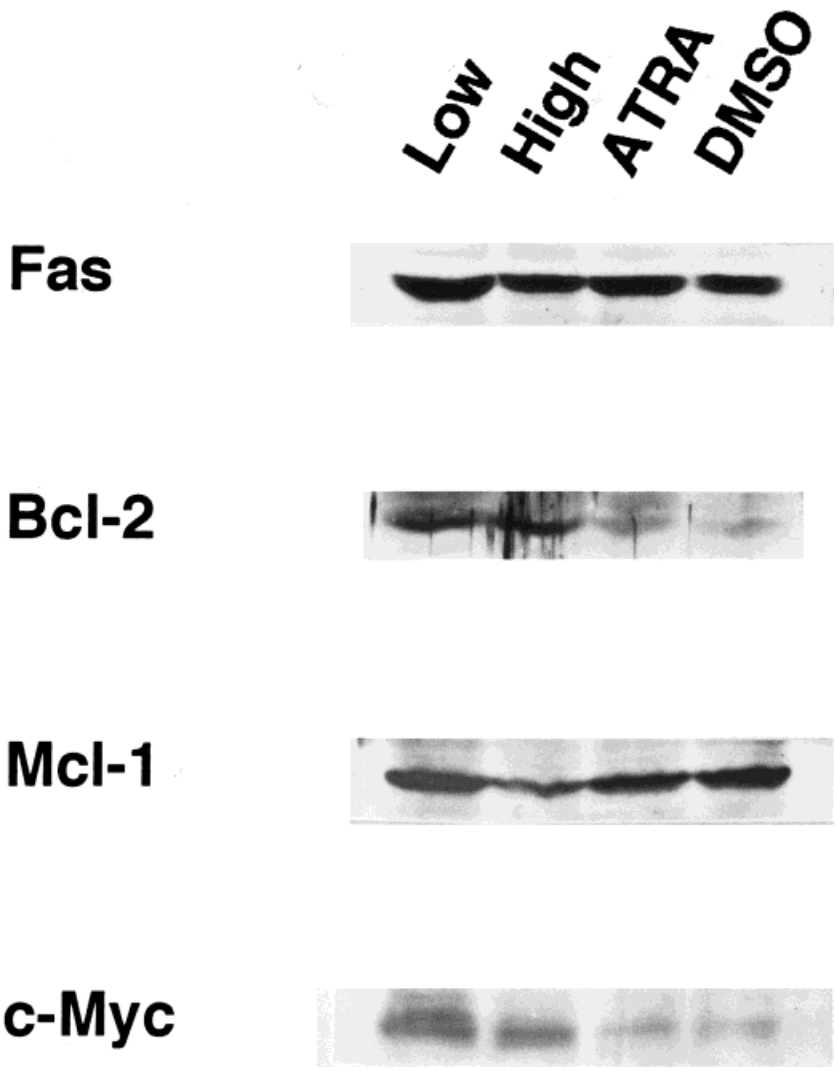

Fig. 8. Expression of apoptosis-related proteins in HL-60 cells cultured under various conditions. Low: cells were harvested at the density of 2.4×10^5 cells/ml. High: cells were harvested at the density of 1.7×10^6 cells/ml. ATRA: cells were cultured with 1 μM ATRA for 4 days. DMSO: cells were cultured with 1% DMSO for 4 days. After harvest, HL-60 cells were solubilized and subjected to Western blotting using a specific antibody for each intracellular molecule.

tion, differentiation, and apoptosis in mammalian cells.

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