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Resistance of HL-60 Promyelocytic Leukemia Cells to Induction of Differentiation and its Reversal by Combination Treatment

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Abstract—*Two sublines of HL-60 cells differing markedly in their ability to undergo differentiation to granulocytes after treatment with retinoic acid (RA), dimethyl sulfoxide (DMSO) and the pyrimidine analog, 5-aza-2′-deoxycytidine (azadCyd) were studied. The sensitive subline (HL-60 S) responded well to 1 μM RA, 1% DMSO and 1 μM azadCyd, showing 89 ± 5%, 46 ± 5% and 29 ± 6% mature nitroblue tetrazolium (NBT)-positive cells, respectively. However, the resistant subline (HL-60 R) showed only modest maturational effects (12 ± 3%, 11 ± 2% and 9 ± 2%, respectively) after treatment with the same agents. Using the HL-60 R as a model for resistance to differentiation induction in the HL-60 cell line, studies were carried out to determine whether the combined use of RA, DMSO and azadCyd could reverse the resistance of these tumor cells to the induction of maturation expressed by the individual agents. When these agents were given in any combination of 2, a minor increase in differentiation induction was detected (13 ± 6% or less NBT-positive cells). However, when all 3 agents were combined (RA + DMSO + azaCyd), resistance was completely reversed (89 ± 7% mature NBT-positive cells). In addition, different degrees of concentration-dependence of each agent in the combination were observed. The RA + DMSO + azadCyd combination caused a maximal accumulation of NBT-positive cells after 72 to 96 hr of incubation. These results show that the lack of competence for induction of differentiation in resistant HL-60 cells can be completely reversed by the above ternary drug combination. However, the mechanism responsible for this synergistic effect must await further elucidation of the molecular mechanisms by which such agents act.*

INTRODUCTION

THE HL-60 leukemia cell line was established by Collins *et al.* from the peripheral blood of a patient with acute promyelocytic leukemia [1]. This cell line responds to a variety of differentiation-inducing agents by ceasing cell division and acquiring characteristics of either mature granulocytes or macrophage-like cells [2]. Granulocytic maturation of HL-60 cells can be induced by compounds such as retinoic acid (RA), dimethyl sulfoxide (DMSO) and also by antimetabolites such as 5-azacytidine, 6-thioguanine or certain anthracylines [3–7].

These various agents appear to exert their maturational effects through different cellular mechanisms. RA acts primarily at the nuclear level, probably through a specific intracellular receptor, modifying gene expression [8, 9]. The mechanism by which polar solvents such as DMSO induce differentiation has not been elucidated, but it is possible that modifications in microviscosity and ion concentrations or ion transport at the plasma membrane level play an important role [10, 11]. In contrast to these 2 agents certain cytotoxic drugs such as 5-azacytidine and its 2′-deoxy derivatives probably act by incorporation into DNA and in inhibition of DNA methyltransferases, leading to change in cytosine methylation patterns and modified gene expression [6].

Considering that differentiation-inducing effects can be triggered by different classes of compounds through separate biochemical pathways, their use in combination warrants study. Recently, there have been some reports describing the synergistic effects of various differentiation-inducing agents in HL-60 cells, at concentrations lower than the optimal concentration for single agents [12]. However, there has been no report on how differentia-

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Abbreviations—RA, retinoic acid; DMSO, dimethyl sulfoxide; azadCyd, 5-aza-2′-deoxycytidine; NBT, nitroblue tetrazolium.

tion-inducing agents in combination might affect those HL-60 cells resistant to differentiation induction.

In our laboratory, we were able to characterize 2 sublines of HL-60 cells which differed markedly in their ability to undergo maturation to granulocytes after treatment with various differentiation inducers [13]. The sensitive subline, early passage cultures from the HL-60 parent line [1], responded to differentiation-inducing agents such as RA or DMSO as previously reported [1, 10, 14]. However, the other subline was highly resistant to the same agents [13]. RA and DMSO inhibited growth in proportion to the effects on differentiation induction, whereas azadCyd inhibited growth equally in the 2 HL-60 sublines.

Taking advantage of this resistant variant of HL-60 cells, we explored the use of combination treatment with RA, DMSO and the pyrimidine analog 5-aza-2′-deoxycytidine (azadCyd). Preliminary results of these experiments have been presented elsewhere [15]. The present study shows how the combined use of different, potentially active, agents in a HL-60 variant can reverse resistance to differentiation induction.

MATERIALS AND METHODS

Cell line and culture conditions

Original HL-60 cells were kindly provided by S.J. Collins and R.C. Gallo [1] and designated HL-60 S because of their susceptibility to the induction of differentiation by retinoic acid or dimethyl sulfoxide. These cells were used in passages 70–90. An HL-60 subline that was unresponsive to the above agents was obtained from L. Smets of the Netherlands Cancer Institute in a late but undetermined passage number. This resistant subline was designated HL-60 R. The latter subline apparently developed spontaneously after numerous passages under ordinary culture conditions. Examination of cells stained with May–Grunwald–Giemsa stain showed that the two sublines were morphologically similar, being comprised of 95% promyelocytes. The common origin of HL-60 S and HL-60 R was confirmed after karyotype analysis. HL-60 cells were propagated as stationary-suspension cultures in RPMI-1640 medium containing 25 mM HEPES (Serva, Heidelberg, F.R.G.) and 10% heat-inactivated fetal bovine seum (Flow Labs., Zwanenburg, The Netherlands) at 37°C in 5% CO_2–95% air atmosphere. Cells were screened for *Mycoplasma* contamination by detection with the fluorescent Hoechst 33258 dye. Cells were passaged twice weekly and initiated at 3×10^5 cells/ml. Experiments were performed with cells in logarithmic growth. HL-60 S grew more rapidly with a doubling time of 32 hr compared to HL-60 R with a doubling time of 39 hr.

Cell growth rate was assessed based on fold increase in cell concentration determined using a hemacytometer. Drug effects were expressed as a percentage of growth relative to control.

Chemicals

DMSO was purchased from Merck (Darmstadt, F.R.G.). 5-Aza-2′-deoxycytidine (azadCyd) was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD) and diluted freshly into medium from concentrated frozen stock solutions in phosphate buffered saline. Drug stability in such frozen storage was reliable as shown by HPLC analysis. RA, nitroblue tetrazolium (NBT) and bovine serum albumin were purchased from Sigma (St. Louis, MO). RA (all *trans*) was stored at −20° C as a stock solution in ethanol which did not interfere with the study of RA.

Induction and assessment of differentiation

Cell differentiation was induced by the addition of RA, DMSO and azadCyd at various concentrations to the culture, alone or in the following combinations: RA + DMSO, RA + azadCyd, DMSO + azadCyd and RA + DMSO + azaCyd. Cells were incubated at 37°C as described above. Cells were counted from day 1 to day 4 and cell viability was determined by trypan blue dye exclusion [16]. At specific days (up to day 4), cell aliquots were suspended at 3×10^5 cells/ml in RPMI-1640 medium (100 μl) and incubated for 30 min with an equal volume of 0.1% NBT, dissolved in phosphate-buffered saline (pH 7.4), with 100 ng TPA/ml as previously described [17]. NBT-positive cells were determined by light microscopy, by counting 200 cells and giving the results as percentages of positive cells/total cell number. NBT assays carried out on day 7 gave similar results.

Data analysis

Percentages of NBT-positive cells from at least 5 different experiments were analysed. Statistical evaluation of differences between values for treated and control was performed using Student's *t*-test for each agent tested in HL-60 S and HL-60 R. Mean values and the standard deviations for each drug alone or in combination were compared in each subline and between sublines HL-60 S and HL-60 R. *P* values of less than 0.05 were considered as an indication of a significant difference between the samples.

RESULTS

The 2 sublines of HL-60 cells differed markedly in their ability to undergo differentiation to granulocytes after treatment with RA, DMSO and azadCyd

Table 1. Effect of RA, DMSO and azadCyd on NBT reduction in HL-60 S and HL-60 R cells

Agent	% of NBT-positive cells		
	HL-60 S	HL-60 R	*P* value
None	7 ± 4	5 ± 3	0.03
RA	89 ± 5	12 ± 3	0.0001
DMSO	46 ± 5	11 ± 2	0.0001
AzadCyd	29 ± 6	9 ± 2	0.001

Values are means ± S.D. at day 4 for at least 5 experiments. RA = retnoic acid, 1 μM; DMSO = dimethyl sulfoxide, 1% (v/v); azadCyd = 5-aza-2′-deoxycytidine, 1 μM.

Table 2. Effect of RA, DMSO and azadCyd in different combinations on NBT rduction in HL-60 S and HL-60 R

Agent	% of NBT-positive cells		
	HL-60 S	HL-60 R	*P* value
RA+DMSO	95 ± 4	18 ± 6	0.0001
RA+azadCyd	94 ± 3	13 ± 2	0.0001
DMSO+azadCyd	72 ± 3	12 ± 3	0.0001
RA+DMSO+azadCyd	96 ± 3	89 ± 7	N.S.

Values are means ± S.D. at day 4 for at least 5 experiments. RA = retinoic acid, 1 μM; DMSO = dimethyl sulfoxide, 1% (v/v); azadCyd = 5-aza-2′-deoxycytidine, 1 μM.

as previously found [13]. For comparison Table 1 describes how the sensitive subline HL-60 S responded well to 1 μM RA, 1% DMSO and 1 μM azadCyd, (29–89% NBT-positive cells), whereas HL-60 R was highly resistant to the same agents (9–12% NBT-positive cells).

When HL-60 S and HL-60 R cells were treated with any of the 2 drug combinations, additive effects were observed (Table 2). In HL-60 R, the increase in the number of NBT-positive cells were only modest (18 ± 6% or less). However, when the RA + DMSO + azadCyd combination was tested in HL-60 R cells a marked increase in the number of mature NBT-positive cells was observed (89 ± 7%). The above percentage of induced cells was in the same range as the highest levels of differentiation induction in HL-60 S cells (96 ± 3%). The differences between these results and those obtained with single agents or any of the 2 drug combinations in HL-60 R were highly significant ($P = 0.0001$).

Cell viability was above 80% after single agent therapy (85 ± 4%); and above 65% for binary and ternary combinations (74 ± 8%). As previously reported [13], DMSO and azadCyd given singly caused a marked inhibition of cell growth in both HL-60 S and R cells (more than 70% inhibition

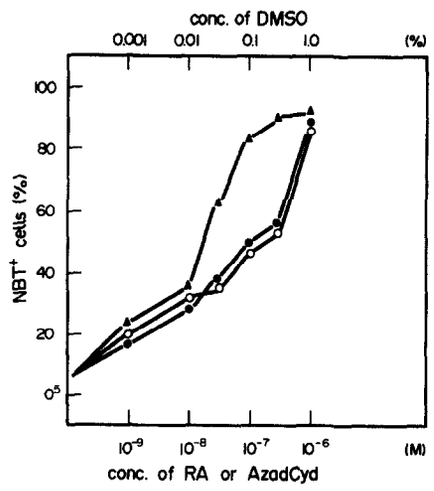

Fig. 1. Concentration-dependence of the induction of NBT-positive cells in HL-60 R cell cultures tested with the RA + DMSO + azadCyd combination. HL-60 R cells were incubated in liquid culture and a series of experiments was performed whereby the concentration of 1 the 3 agents was decreased as the other 2 agents were held at the initially-tested concentration, i.e. RA (1 μM); DMSO (1%); and azadCyd (1 μM). For each experiment, duplicate samples (100 μl) of the cell suspension were tested for the ability to reduce NBT dye. ○ RA concentration was varied; ● DMSO concentration was varied; ▲ azadCyd concentration was varied.

relative to control). RA alone caused a marked growth-inhibitory effect in HL-60 S cells, but only a modest effect in HL-60 R cells (78% vs. 29%, respectively). Binary or ternary combinations caused a marked growth-inhibitory effect on cells from both sublines.

In order to evaluate the concentration dependence of each agent in the RA + DMSO + azadCyd combination, a series of experiments was performed with HL-60 R cells, whereby the concentration of 1 agent was varied as the other 2 agents were held at the initially-tested concentration. As RA concentration was varied from 1 μM to 10 nM, the percentage of NBT-positive cells decreased from 89 ± 7% to 38 ± 5%; and when DMSO was lowered from 1 to 0.05%, the percentage of mature NBT-positive cells declined in proportion to 35 ± 2%. However, a reduction of azadCyd concentration from 1 μM to 50 nM led to a limited decrease in the number of NBT-positive cells to 67 ± 6% (Fig. 1).

The time dependence in the appearance of NBT-positive cells after treatment of HL-60 R cells with the RA + DMSO + azadCyd combination was also studied. Daily measurements of the number of NBT-positive cells were performed, revealing maximal effects after 72 hr of incubation (Fig. 2).

DISCUSSION

The fact that maturational effects can be induced in HL-60 cells by a variety of unrelated compounds suggests that there must be more than 1 mechanism

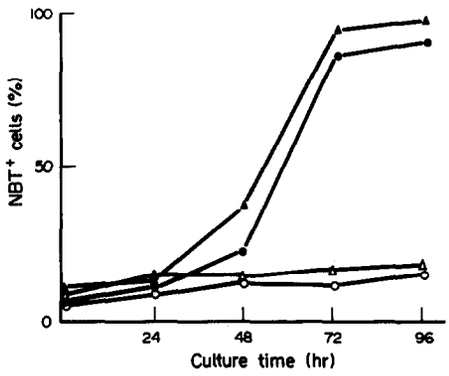

Fig. 2. Kinetics of accumulation of NBT-positive cells in HL-60 S and HL-60 R cell cultures treated with RA + DMSO + azadCyd. Cells were incubated in liquid culture with no drug or RA (1 μM), DMSO (1%) and azadCyd (1 μM). At 24-hr intervals, duplicate samples (100 μl) of the cell suspension were tested for the ability to reduce NBT dye. △ control HL-60 S cells; ○ control HL-60 R cells; ▲ RA + DMSO + azadCyd-treated HL-60 S cells; ● RA + DMSO + azadCyd-treated HL-60 R cells.

by which differentiation induction may be triggered [5, 6, 7, 14, 17, 18]. RA probably modifies cell proliferation and induces differentiation by altering gene expression [19]. However, its mode of action for eliciting cellular programs regulating growth or differentiation remains obscure [8, 9].

Regarding the mechanism by which polar solvents such as DMSO induce tumor cell maturation, there is evidence that changes in membrane ion concentrations and ion transport, and modifications in the structure of the cell membrane may play an important role [20]. Compared with DMSO, RA leads to a more rapid decline in clonogenic capacity of HL-60 cells and also to a more complete accumulation of mature segmented neutrophils [4, 14]. Studies with HL-60 cells have also demonstrated a preferential commitment during the S or the S–G_2 interphase after treatment with RA [21]. In contrast, DMSO appears to lead to a similar effect without specific relation to the cell cycle pattern [4, 21].

Cytotoxic drugs that inhibit DNA replication such as cytosine arabinoside or 6-thioguanine seem to induce differentiation via other pathways [5, 22, 23]. Interestingly, maturational effects of these agents can be demonstrated at non-cytotoxic concentrations [22, 23]. Similarly, DNA-hypomethylating agents such as 5-azacytidine or azadCyd are also differentiation inducers in HL-60 cells, probably through alterations in gene expression [6]. The latter drug has been recently studied by us [13, 15, 24, 25].

In the present study, clear differences in differentiation induction after treatment of HL-60 cells with RA, DMSO and azadCyd were detected either between different agents in each of the 2 sublines or between the same agent in HL-60 S and HL-60 R cells. In HL-60 S, all 3 agents were capable of inducing cell differentiation, although the effect was more marked after RA or DMSO than azadCyd. In HL-60 R, the above agents had only minor maturational effects.

When cells from both sublines were treated with any of the binary combinations, no advantage or partial additive effects were observed. It is possible that the increase in the number of NBT-positive cells by the addition of the second agent was dependent on the induction of cells that failed to respond to the first differentiation inducer, as previously reported [4].

Interestingly, when the 3 agents were given in combination to HL-60 R cells, a highly significant increase in the number of mature NBT-positive cells was observed. The RA + DMSO + azadCyd combination raised the percentage of induced cells to almost 100% as opposed to 18 ± 6% or less NBT-positive cells detected after any of the combinations of 2 agents. The RA + DMSO + azadCyd treatment was equally effective in both sublines of HL-60 cells, characterized by the complete reversal of resistance to differentiation induction in the resistant variant subline.

Our study also suggests that the above agents may show different degrees of concentration-dependence for the maximal maturational effects. Decreases in RA or DMSO concentrations were more critical for the maintenance of the synergistic effects. However, a reduction of the azadCyd concentration within similar ranges led to a more limited decrease in the effects of the combination. In addition, the kinetics of accumulation of induced cells followed the same pattern observed when sensitive HL-60 cells were treated with RA alone or in combination with DMSO, azadCyd or both (not shown).

In conclusion, our findings show that the lack of competence for induction of differentiation can be reversed in HL-60 cells using at least 3 potential inducers in combination. Further studies are required to examine the changes in cell surface antigens and other biochemical and functional markers to better characterize this form of induced differentiation.

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