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INDUCTION OF GRANULOCYTIC MATURATION IN HL-60 HUMAN LEUKEMIA CELLS BY FREE RADICALS: A HYPOTHESIS OF CELL DIFFERENTIATION INVOLVING HYDROXYL RADICALS

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Tumor cells usually contain lower superoxide dismutase (SOD) activity than differentiating cells, suggesting the involvement of oxygen free radicals in cell maturation. The effects of a system known to produce the $OH^{\cdot}$ radicals were tested on HL-60 cells cultured under optimum conditions for 96 hr. Hydroxyl radicals were generated by a Fenton reaction, involving an ADP-Fe^{2+} (or ATP-Fe^{2+}) complex and H_2O_2. Changes induced by $OH^{\cdot}$ were compared to the effects of DMSO-induced differentiation of HL-60 cells. Cell numbers, viability, thymidine incorporation, TPA-induced NBT reduction and propidium iodide staining in flow cytometry were determined. The $OH^{\cdot}$ generating system inhibited the growth and thymidine incorporation of leukemic cells in a manner dependent on the dose of added H_2O_2 (from 0.005 to 0.05 mM). In addition, an increasing proportion of the treated cells displayed signs of cell differentiation. In DMSO-treated cells, SOD and catalase activities increased after 6 days of culturing. The results show that a portion of the $OH^{\cdot}$ free radicals derived from H_2O_2, produced by the action of SOD, may be a necessary prerequisite for differentiation, whereas an overproduction of $OH^{\cdot}$ causes cell lethality or aging. We suggest that $OH^{\cdot}$ free radicals may have a more complex role in cell physiology than simply causing oxidative damage.

KEY WORDS: $OH^{\cdot}$ free radicals, HL-60 leukemia cell line, DMSO-induced differentiation of HL-60 cells, SOD, catalase, cell differentiation

INTRODUCTION

A number of chemically distinct compounds induce differentiation of the HL-60 human promyelocytic leukemia cell line,[1,2] resulting in neutrophilic morphology and phagocytic functions. For example, dimethyl-sulfoxide (DMSO);[3–7] phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA);[8–10] actinomycin D;[11] retinoic acid;[6,12,13] nucleosides, db-cAMP and methotrexate;[14,15] 1,25-dihydroxy-vitamin D_3;[16–19] anthracyclines;[20,21] 6-thioguanine;[22] γ-interferon;[23] dimethylformamide[24] and histamine[15]. During differentiation, promyelocytic HL-60 cells stop growing, become much smaller in size[12,25] and acquire the ability to form reactive

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oxygen species as shown by the appearance of the TPA-stimulated respiratory burst, detected by the nitro blue tetrazolium (NBT) reaction and other methods.[26-28]

In spite of much experimental data, no commonly accepted mechanism of action has been proposed for the compounds known to induce differentiation. However, some data indicate that the earliest effects in HL-60 cells of differentiation-inducers, such as phorbol esters and retinoic acid is a rapid down-regulation of the surface transferrin receptor.[10,25,29,30] Whereas, others have concluded that the early decline in transferrin receptor levels precludes its regulation as a consequence of the decrease in proliferation, but rather implicates its role in the programmed cessation of growth when the terminal differentiation occurs[31]. According to Barker and Newburger,[13] transferrin receptor gene expression becomes considerably less, nine to twenty-four hours subsequent to the treatment of HL-60 cells with retinoic acid induced differentiation. Furthermore, it has been shown that bryostatin (an activator of protein kinase C) causes a rapid down-regulation of the surface transferrin receptors, but fails to induce differentiation of HL-60 cells, that is, the TPA-induced differentiation of HL-60 cells is independent of transferrin availability.[32]

Another aspect of the problem of cell differentiation is that non-differentiated cells and tumor cells usually display lower SOD activities than differentiated cells. Furthermore, SOD activity increases dramatically in a variety of tumor cell systems when they undergo differentiation.[33-35] However, after reaching a certain level of differentiation, both SOD activity and the SOD mRNA production start to decrease in various cell lines.[24,36,37] These data show that a transient increase in SOD activity may be considered a prerequisite for the onset of cell maturation. It has been hypothesized that this phenomenon may be explained by assuming that part of the $OH\cdot$ free radicals deriving from H_2O_2 (the latter produced by SOD), may play a physiologically useful role in the process of cell maturation.[38,39]

Hydroxyl radicals have long been known as cross-linking agents,[40-43] and it has been shown that these radicals when generated under physiologically relevant chemical conditions (e.g., at pH 7.2 by the iron-mediated heterolysis of H_2O_2 i.e., the Fenton reaction[44]) are able to react readily with all amino acids.[45,46] A hypothesis has been proposed which suggests that $OH\cdot$ radicals may be necessary for completion of the maturation processes in cells through the formation of useful intermolecular cross-links.[38,39]

To test this hypothesis we have examined the direct effect of $OH\cdot$ radicals on cell differentiation in well-defined cellular models. Similar experiments using a K562 human leukemia cell line[48] have previously been performed in our laboratory.[47] Hydroxyl radicals were found to produce a considerable erythroid differentiation of K562 cells, using haemoglobin synthesis as a measure of "normalization" of the malignant cells. These studies have now been extended to other cell types to investigate how general the effect of $OH\cdot$ is on cell differentiation. The present paper describes the results obtained on HL-60 human promyelocytic leukemia cells[1,2] using well known markers of differentiation induction.

MATERIALS AND METHODS

Materials

The following products were used: 12-O-tetradecanoylphorbol-13-acetate (TPA), propidium iodide, RNAse, SOD, catalase, xanthine oxidase, xanthine, folin-phenol

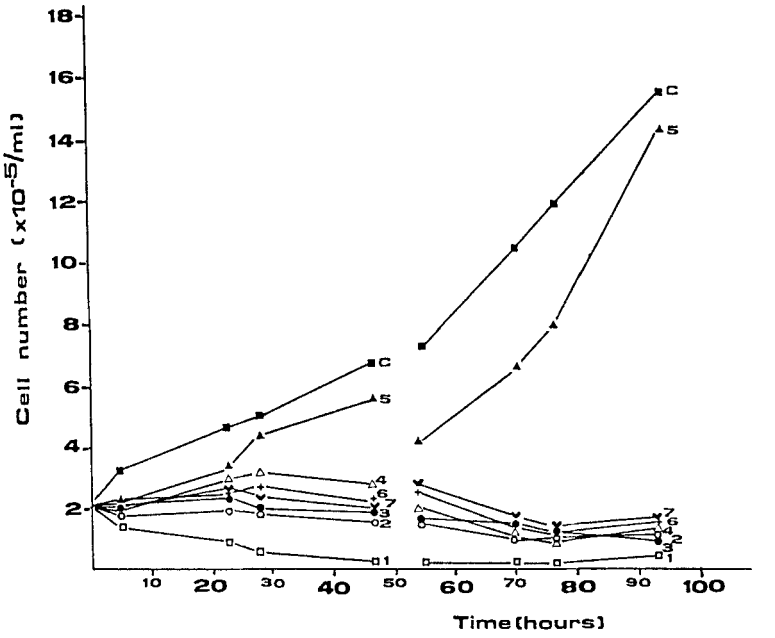

FIGURE 1 Cell growth of HL-60 cells during 96 hrs of culturing. C indicates the control system, whereas numbers 1–7 designate the experiments described and numbered identically in Table 1. Data are from one representative experiment. The renewal of the medium after 48 hrs induced some small changes in cell numbers in each culture.

reagent, SDS and Triton-X-100 from Sigma; (6-^{3}H)thymidine from Amersham International. All other reagents were from the Reanal Budapest with the exception of fetal bovine serum which was the product of Human Institute for Serobacteriological Production and Research, Budapest-Gödöllő, Hungary.

Cell culturing

The HL-60 human acute promyelocytic leukemia cell line was cultured under standard conditions, i.e., in RPMI 1640 medium supplemented with 50 IU/ml of penicillin, 50 μg/ml of streptomycin and 20% fetal bovine serum in a volume of 20 ml, kept in 75 cm^2 tissue culture flasks (Greiner) at 37°C in a humidified, 5% CO_2 atmosphere. The initially seeded cell number was 2×10^5 cells/ml. Cell numbers were determined with a hemocytometer twice daily in regular time intervals up to 96 hrs, as shown in Figure 1. Only viable cells (trypan blue exclusion) were counted. Non viable cells amounted to only a few percent in each culture, and were not counted.

Experimental interventions

Hydroxyl radicals were generated in the cultures by the method of Floyd and Lewis[45,49] which consists of the addition of ADP-Fe^{2+} (or ATP-Fe^{2+}) complex and H_2O_2. These components were added to various experimental systems in quantities

TABLE 1
The composition of the HL-60 cell culture systems tested in the experiments. Final concentrations of ADP or ATP were 1 mM, of Fe^{2+} was 0.05 mM in each experiment. H_2O_2 concentrations (mM) varied as indicated.

Group no.	Added substances			
	ADP-Fe^{2+}	ATP-Fe^{2+}	ADP	H_2O_2
Control	-	-	-	-
Control + DMSO	-	-	-	-
Exp. 1.	+	-	-	0.05
Exp. 2.	+	-	-	0.01
Exp. 3.	+	-	-	0.005
Exp. 4.	+	-	-	-
Exp. 5.	-	-	-	0.01
Exp. 6.	-	+	-	0.01
Exp. 7.	-	-	+	-

Notes: the nucleotide-Fe^{2+} complex was prepared immediately before the addition to the culture medium. Additions were performed at 0 and 48 hrs of culturing, except the DMSO which was added only at 0 time.

as shown in Table 1. During the experiments, the HL-60 cells were cultured under otherwise standard conditions for 96 h. After the first 48 hours of culturing, the cells were pelleted by centrifugation and resuspended in the same volume of fresh RPMI 1640. The experimental cell groups were then treated with identical freshly prepared reaction mixtures as shown in Table 1 for another period of 48 hours. Control cultures were run in parallel with each experiment. All the experiments were repeated at least 3-times. The determination of cell number and thymidine incorporation assay were performed twice daily. The cell staining and flow cytometric analysis was accomplished at the end of the 4-day culture period.

Induction of granulocytic maturation of HL-60 cells was achieved by the addition of 1.25% DMSO[26]. DMSO-induced differentiation becomes complete in 6 days, therefore, all measurements with this compound were performed after a 6-day-period of culturing.

Assay of the TPA-stimulated NBT reduction

This assay[26,28] was performed at the end of the 96 hrs culturing, except in the DMSO-induced system where 6 days culturing was used. Cell samples (10^6/ml) suspended in 1 ml of phosphate-buffered saline (PBS), pH 7.2 (containing 136 mM NaCl, 2.6 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$ and 0.5 mM $MgSO_4$) were incubated with an equal volume of 0.2% NBT dissolved in PBS in the presence, or absence, of 100 ng/ml of freshly diluted 12-O-tetradecanoylphorbol-13-acetate (TPA) at 37°C for 25 min.[12] At least 200 cells from each sample were counted to determine the percentage of cells containing intracellular blue-black formazan deposits in Wright-Giemsa stained preparations.

Thymidine incorporation assay

This assay was performed twice daily in samples taken at regular time intervals up to 96 hrs, as shown in Figure 2. The rate of DNA synthesis was measured according to Becton and Roberts[50] by the incorporation of (6-^{3}H)thymidine. For this purpose, 100 μl of cell suspension was plated in triplicate into 96-well microtiter plates.

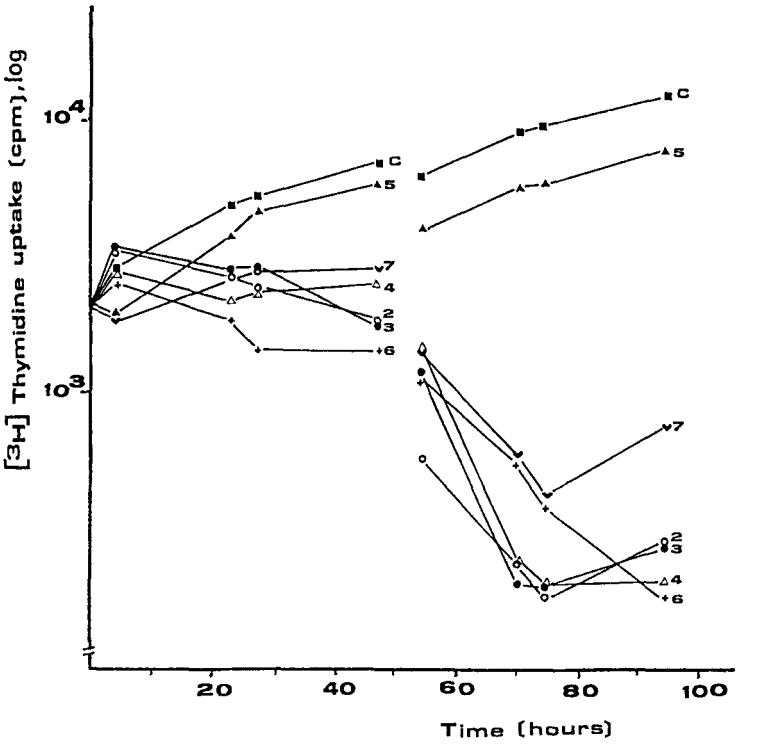

FIGURE 2 (^{3}H)thymidine uptake in HL-60 cells during 96 hrs of culturing. C indicates the control system, whereas numbers 1-7 designate the experiments described and numbered identically in Table 1. Data are from one representative experiment. Note the logarithmic scale on the vertical axis. The renewal of the medium after 48 hrs induced some small changes of DNA synthesis in each culture.

Pulse-labelling was then performed with 0.2 μCi/well (^{3}H)thymidine, followed by culturing for 4 hrs at 37°C in a humidified incubator in the presence of 5% CO_2. Cells were then harvested, collected on filter paper, and dried. The incorporated thymidine was measured by liquid scintillation counting.

Flow cytometry determining the cell cycle phase

This assay was performed at the end of 96 hrs culturing, except in the DMSO-induced system where 6 days culturing was used. The cells were washed in PBS and fixed in 70% ethanol at 0°C.[51] Fixed cells were stable for at least 2 weeks at 4°C. The cells were rinsed with PBS and treated with 1 mg/ml of RNAse in PBS at 37°C for 30 min, followed by staining with 50 μg/ml of propidium iodide. The cells were filtered through a 37 μm nylon mesh to remove aggregates prior to flow analysis. The cells stained with propidium iodide were analyzed using a flow cytometer (FACS III, Becton Dickinson FACS Systems, USA) with a 488 nm exciting laser beam and recording the fluorescence intensity as a measure of the DNA content, as described by Blair et al.[5] At least 10 thousand cells were counted in each sample. The DNA histograms obtained were analyzed to determine the distribution of cells in the G_0/G_1, S and G_2+M phases of the cell cycle using a suitable computer program.

TABLE 2
Absolute viable cell numbers and their percental comparison, with the relative (^{3}H)thymidine incorporation in HL-60 cells at the end of 96-hour culturing, (for DMSO 6 days) (mean ± S.D. of 3 runs)

Experiments	Cell number 10^4/ml	Cell number %	(^{3}H)thymidine incorp. (%)
Control	183.8 ± 22.7	100 ± 12.3	100 ± 8.3
Cont. + DMSO	31.9 ± 7.0	17.4 ± 3.8	n.m.
Exp. 1	1.8 ± 1.6	1.0 ± 0.8	n.m.
Exp. 2	14.8 ± 2.1	8.0 ± 1.1	2.8 ± 0.8
Exp. 3	10.4 ± 4.6	5.6 ± 2.5	2.3 ± 0.3
Exp. 4	13.0 ± 3.7	7.0 ± 2.0	2.1 ± 0.3
Exp. 5	145.7 ± 1.9	79.2 ± 1.0	71.6 ± 8.2
Exp. 6	10.3 ± 4.6	5.6 ± 2.5	1.7 ± 0.4
Exp. 7	12.3 ± 2.9	6.6 ± 1.5	7.5 ± 1.0

Notes: All the experimental results differ significantly from controls at $p < 0.001$, except the Experiment 5 where the thymidine incorporation rate differs at $p < 0.005$. Starting, seeded cell number at 0 time was 20×10^4/ml in all cultures. n.m.: not measured.

Protein content and enzyme determinations

The cells were washed with PBS and disrupted by sonication. Protein content was determined by the method of Markwell *et al.*[52] Enzyme determinations were only carried out in control and DMSO treated cultures at the end of the 6 days culture period as detailed below. In these experiments the cultures were continuous without any intervention for 6 days.

Superoxide dismutase (SOD). The method of Flohé and Ötting[53] was used for total SOD activity. Here, the reduction rate of cytochrome c by superoxide radicals is detected at 550 nm using the xanthine-xanthine oxidase system as a source of superoxide radicals.

Catalase (CA) was determined by the method of Gaunt and De Duve.[54] Cell suspensions in the presence of 0.1% of Triton X-100 were incubated for 30 min at 25°C with known concentration of H_2O_2 solution containing 10 mM imidazole buffer, pH 7.0. The reaction was stopped by the addition of titanium oxysulfate. The remaining H_2O_2 was measured spectrophotometrically at 405 nm.

RESULTS

Cell proliferation

The number of viable HL-60 cells, cultured with various experimental additions, was counted at different intervals up to 96 h as shown in Figure 1.

Cell growth was rapid in control cultures during the observation period, with cell numbers showing a near linear increase. During the 96 h culture period the cell numbers in the controls increased an average of almost tenfold (Table 2). The increase was around 1.5-fold (in 6 days) in the presence of DMSO, and was completely abolished in experiments 1–7, with the exception of experiment 5, where only H_2O_2 was added to the culture, and which caused an increase in the cell number of more than 7-fold in 96 hrs (Table 2). In all the other experimental systems as shown in Figure 1, this resulted in a cell number slightly below the initial value. In experiment 1, where the highest concentration of H_2O_2 was added together with the

ADP-Fe^{2+} complex, the loss of cell number was so great that this experiment could not be used for further analyses. Lower concentrations of added H_2O_2 (experiments 2 and 3) yielded a negligible cell loss during the first 48 hrs of culturing, and more (up about 50%) during the second half of the observation period. A similar degree of inhibition was observed when ADP-Fe^{2+} complex was added alone (experiment 4). When the ATP-Fe^{2+} complex plus H_2O_2 (experiment 6) was tested, the same level of growth inhibition occurred, as in experiment 2 where ADP was used on place of ATP. When ADP was added alone without iron and hydrogen peroxide (experiment 7), it still caused a significant inhibition of growth.

DNA synthesis

The reactants listed in Table 1 resulted in very strong inhibition of the incorporation of (^{3}H)thymidine into HL-60 cells (Figure 2). The rates of inhibition in various experimental groups followed the patterns found in cell growth inhibition. The 0.05 mM H_2O_2 concentration (experiment 1) decreased thymidine incorporation below 1000 cpm, which also caused the statistical scatter of the results to increase. Therefore, this model could not be used further. Table 2 compares the effects of the reactants on DNA synthesis, and on cell number. These two parameters appear to parallel each other.

TPA-induced NBT reduction assay

It has previously been established that the morphologically mature, differentiated HL-60 cells, capable of phagocytosis, reduce NBT well when stimulated by TPA, whereas the promyelocytic, immature HL-60 cells do not.[12,26] This means that TPA evokes the respiratory burst in these cells, if they undergo the maturation process. NBT is a water-soluble dye taken up by the cells, and its reduced form is an insoluble intracellular blue-black formazan in the mature HL-60 cells. In our studies, this reaction was always performed on two aliquots of the same cell sample; one treated with TPA, and the other not. Thus, one can be assured that only the TPA-induced NBT reduction is considered here. The results are summarized in Table 3, showing the percentage of cells stained black with reduced NBT.

Approximately 3% of the control HL-60 cells were stained positively in the NBT reduction assay, indicating that spontaneous differentiation occurs only very rarely. With the exception of the sole H_2O_2 treatment (experiment 5), where the difference between treated and controls was not significant, all the other treatments induced differentiation of HL-60 cells to a highly significant extent. These were comparable to, or even higher, than the DMSO-induced NBT positive values obtained after 6 days (Table 3).

Flow cytometric results

The flow cytometric analysis was performed following 96-hours incubation. The results (Table 4, Figure 3) show the distribution of cells according to their DNA content, a basis for determination of cell cycle phase at the time of fixation. Figure 3 shows some characteristic curves of a single but representative experiment. Integration of the areas below the peaks gives the distribution of cells in various phases of the cell cycle. Table 4 summarizes the results in controls and various experimental models. In control cultures, about one third of the cells are in the S and G_2+M

TABLE 3
TPA-induced NBT staining of HL-60 cells at the end of the 96-hour culture period, (for DMSO 6 days) (mean ± S.D. of 3 runs).

Experimental groups	NBT-reducing cells (%)
Control	3.4 ± 2.3
DMSO	78.2 ± 9.7
Exp. 1	n.m.
Exp. 2	81.3 ± 6.9
Exp. 3	86.4 ± 4.4
Exp. 4	69.2 ± 15.4
Exp. 5	7.5 ± 5.8
Exp. 6	80.0 ± 7.3
Exp. 7	91.0 ± 5.4

Notes: the differences between the control and all experimental groups are statistically significant at $p < 0.001$, except the Experiment 5 which is not different from the control. n.m.: not measured.

TABLE 4
Distribution of HL-60 cells in various phases of the cell cycle based on flow cytometric determination of the DNA contents at the end of the 96-hour culture period, (for DMSO 6 days) (mean % ± S.D. of 3 runs)

Experiments	G_0/G_1	S	G_2+M
Control	65.9 ± 6.6	19.8 ± 6.8	14.1 ± 2.4
DMSO	91.6 ± 2.3	3.7 ± 1.5	4.6 ± 0.9
Exp. 1	n.m.		
Exp. 2	94.4 ± 1.4	4.1 ± 1.7	1.4 ± 1.3
Exp. 3	94.8 ± 1.0	2.6 ± 0.5	2.6 ± 0.5
Exp. 4	95.1 ± 2.2	3.2 ± 1.5	1.6 ± 0.8
Exp. 5	63.7 ± 5.9	23.2 ± 6.0	13.1 ± 0.1
Exp. 6	91.1 ± 5.1	5.0 ± 2.6	3.8 ± 2.4
Exp. 7	87.5 ± 2.3	6.8 ± 0.4	5.6 ± 1.9

Notes: differences in the G_0/G_1 column against the control are significant at $p < 0.001$, except Experiment 5 which is not different from the control. n.m.: not measured.

phases. With DMSO, and H_2O_2 plus ADF-Fe^{2+} treatment, with the exception of experiment 5, a significant decrease in the proportion of cells in these phases occurred, whereas most of the cells were arrested in the G_0/G_1 phase. The extent of the accumulation of cells in the G_0/G_1 phase of the cell cycle was statistically significant. There was, however, no significant difference in the percentage of G_0/G_1 phase between the cells of experiment 5 and the control cells.

Enzyme levels in DMSO-treated cultures

Total SOD and catalase activities found in control and DMSO-treated HL-60 cells after 6 days of culturing are shown in Tables 5 and 6, respectively. These activities can be expressed either per mg protein or per cell number. The two ways of presentation reveal apparently different trends. However, in reality there is no contradiction, as explained below.

After 6 days of culturing, i.e., when the differentiation was already completed, the SOD activity of the DMSO-treated HL-60 cultures was about 23% higher, if expressed per mg protein (Table 5). However, it is not a contradiction that the SOD activity when expressed on a per cell basis, showed a decrease of about 44%

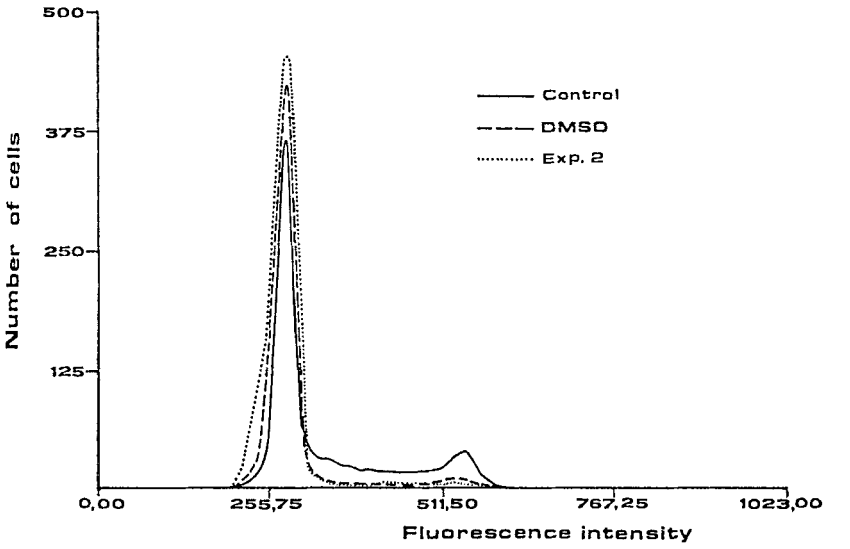

FIGURE 3 Flow cytometric DNA distribution histograms in control, DMSO treated and Experiment 2 in HL-60 cells. For more details see the text.

TABLE 5
Total superoxide dismutase activities in control and DMSO treated HL-60 cells, both cultured for 6 days (mean ± S.D. of 3 experiments).

Experimental groups	Superoxide dismutase (ng/mg protein)	Superoxide dismutase (ng/10^6 cells)
Control	1788.8 ± 126.1	481.5 ± 32.6
DMSO	2192.5 ± 126.9	270.0 ± 15.8
Change in	+22.6	−43.9

Note: Values of DMSO treated group are significantly different from the control at $p < 0.001$.

TABLE 6
Catalase activities in control and DMSO treated HL-60 cells, both cultured for 6 days (mean ± S.D. of 3 experiments).

Experimental groups	Catalase (U/mg protein)	Catalase (U/10^6 cells)
Control	118.4 ± 13.7	32.3 ± 3.6
DMSO	164.5 ± 8.8	19.9 ± 1.0
Change in %	+38.9	−38.4

Note: Values of DMSO treated group are significantly different from control at $p < 0.001$.

(Table 5). This is because the differentiated cells become 50% smaller in diameter, i.e., about 2^3 = 8-fold smaller in their volume, i.e., they lose seven eighths (about 88%) and maintain only one eighth (about 12%) of their volume. This results in the cells having about 56% of their original SOD activity per cell in about 12% of the volume in the differentiated state. This means a 4.7-fold increase of the relative SOD activity per differentiated cell after 6 days of culturing.

With regard to catalase activity, it shows an increase of about 39% on a per mg protein basis, but decreases about 38% on a per cell basis in the DMSO-treated cells (Table 6). Following the same logic with regard to cell size, 62% of the original catalase activity per cell remains in about 12% of the cell volume, i.e., the relative catalase activity per cell increases about 5.1-fold.

DISCUSSION

The interpretation of the present results rests on well established scientific facts summarized below. In addition, we speculate around the hypothesis we propose.

1. The role of ADP or ATP in the reaction is probably not specific. Either nucleotide chelates divalent iron and slows its rate of autoxidation, which would otherwise take place rapidly at physiological pH values and oxygen pressure. This allows the chelated Fe^{2+} to remain available to react with H_2O_2 in a Fenton reaction. The ADP-iron and ATP-iron complexes react with H_2O_2 to generate OH· free radicals as shown by spin trapping and other methods.[45,49,55,56] Although OH· production by this reaction is rapid in vitro,[56] the addition of ADP-iron to cell cultures may cause a longer-lasting effect by two mechanisms: (i) Although the iron is oxidized in the complex,[54] its trivalent form also participates in the production of OH· free radicals through the Haber-Weiss reaction,[57] with an efficiency about 5 times lower.[49] (ii) Because of the presence of ascorbate in the culture, the oxidized iron becomes reduced to Fe^{2+}, and the continuous production of H_2O_2 during the cellular respiration will be able to maintain a more or less constant flux of OH· radical production. In other words, one can assume that the addition of the ADP-iron complex induces a redox cycle which may influence the cells for a longer time.

2. Hydroxyl radicals react rapidly with adjacent chemical moieties and are able either to alter the molecular conformation or cause intermolecular cross linking.[40–43] The altered conformation and cross-linking are usually considered to be damaging. However, they can also be useful, since they may contribute to the stabilization of subcellular structures.[38,39] In other words, the assembly of molecular components into membranes, cytoskeleton, etc. may be facilitated by a flux of OH· free radicals. This hypothesis proposes the following possibilities: If the OH· radical flux is moderate, the advantageous cross-linking effects of these radicals may be predominant, and the cells may be able to respond to the relatively small damaging effects by resynthesis of the altered compounds. However, when there are extremes from this equilibrium, the cells either may not become mature (if too few radicals are available), or age too fast (if too many radicals are formed), or may even die.

The present experiments appear to support such a working hypothesis. Namely, if we assume that the blast-type, ever dividing HL-60 cells are deficient in OH· radicals or a species with similar reactivity, a certain increase in the production of the latter should cause differentiation of the cells, whereas an overproduction of OH· radicals should be lethal to them. Interestingly, in our experiments, production of OH· radicals in a relatively low range caused an almost complete differentiation, whereas a greater flux of these radicals in experiment 1 caused severe cell damage, including death.

It should be noted that even an incomplete addition of the components of the Fenton reaction mixture could produce inhibitory effects on cell growth and DNA synthesis, and a stimulation of cell maturation. This does not contradict our hypothesis, since certain amounts of all the added components are physiologically

present in the cells or in the culture medium. Nevertheless, one can ask why don't the control cells differentiate? The answer to this question may be related to the following points:

(i) Only a marginal inhibition of growth was observed in experiment 5 where 0.01 mM H_2O_2 was added. Since the thymidine incorporation in this experiment decreased slightly, but significantly in statistical terms (Table 2), one can assume that even this small increase in the cellular H_2O_2 starts to shift the cells toward differentiation. The fact that the same concentration of H_2O_2 was very efficient in experiments 2 and 6, where complexed iron was also added, indicates that possibly the balance between the available intracellular divalent iron and the flux of H_2O_2 is not optimal to maintain a yield of OH· radicals sufficiently high for differentiation of the HL-60 cells.

(ii) When we added only ADP-Fe^{2+} (experiment 4), it could obviously react with the physiologically produced H_2O_2 in the cells. Even ADP added alone (experiment 7) produced significant effects, since it chelated divalent or trivalent iron from the culture medium. If it then enters the intracellular space, its ability to delay the autoxidation of Fe^{2+}, formed by naturally present reductive mechanisms, such as ascorbate[58,59], could increase OH· radical yield in the cells. The similar effect of ADP- or ATP-iron complexes suggests that the induction of maturation is not specific to the nucleotide itself, but results from its iron-chelating properties. It should, also be noted that while the increase of ADP concentration in the cells stimulates cell respiration, ATP has an opposite effect, i.e., the idea that additions of nucleotides act simply through the stimulation of oxygen consumption does not appear to be valid.

Our results show that the increased production of OH· free radicals by the added components of a Fenton reaction, up to a certain level, may be able to induce complete differentiation of HL-60 promyelocytic leukemia cells. The question arises, however, whether the OH· radical production may, or may not, be an important step in the differentiation process induced by other compounds such as DMSO.

In order to tentatively answer this question, we have measured SOD and catalase activities in the HL-60 cells both in control and DMSO-treated cultures. Both enzymes show a significantly increased level in the differentiated state, as compared to the controls (Tables 5 and 6). When one considers that both the SOD activity and the mRNA of SOD are usually decreasing by this time in differentiated cells,[24,36,37] it seems that during the decisive, initial phases of differentiation, an even higher SOD activity was present in the DMSO-treated cells. An essential property of all SODs is, as shown by pulse radiolytic techniques,[60,61,62] that SOD competes very efficiently with the reductive reactions of the available O_2^-· radicals. Therefore, in the presence of SOD, O_2^-· radicals are more rapidly transformed into H_2O_2, than in its absence. At the same time, because a number of slower reactions of O_2^-· radicals cannot take place in the presence of elevated SOD levels, relatively more H_2O_2 is formed, compared to spontaneous dismutation. Such a situation is consistent with the assumption that the increased SOD activity may be responsible for the induction of differentiation by DMSO, through an increased production of H_2O_2.

There is, however, an apparent contradiction. Namely, it is well known that DMSO is an efficient scavenger of OH· free radicals in vitro. It may seem absurd to expect an increase in OH· radical production from a scavenger of the same radical. Nevertheless, one may consider several points: First, it is important to note that 1.25% DMSO is given to the cultures only once at the beginning, and complete differentiation of the cells is reached after 6 days. Therefore, it is possible that an

initial lowering of the available OH· radical flux in the cells may switch on some regulatory processes which can stimulate OH· radical production after the DMSO is completely metabolized in the culture medium. One may also assume that DMSO itself, or various metabolic products of it, exert effects on oxidative cell metabolism by possibly changing permeabilities for substrates in cell and mitochondrial membranes.

Apart from the above considerations, our present experiments show that DMSO-induced differentiation of HL-60 cells is accompanied by a considerable increase in SOD activity, and a similar phenomenon was observed when the differentiation of the same cell line was induced by DMF.[24] This may result in a higher production of H_2O_2 compared to non-differentiated blast cells. This is consistent with the notion that increased OH· radical production may even be involved in the DMSO-induced differentiation of HL-60 cells. A detailed product analysis may offer further insight into this point. It may be relevant to mention here that recent publications[63-65] have drawn attention to the possibility that oxygen radicals may act as important messenger, signal and trigger molecules in biological systems.

We believe that our present experimental findings support our hypothesis that part of OH· free radical production is necessary for cell maturation and differentiation.

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