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Induction of the Differentiation of HL-60 Promyelocytic Leukemia Cells by L-Ascorbic Acid

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The present study was undertaken to examine the effect of L-ascorbic acid (LAA) on the growth of HL-60 promyelocytic leukemia cells, besides induction of apoptosis. LAA ($\geq 10^{-4}$ M) was found to markedly inhibit the proliferation of HL-60 in liquid culture and clonogenicity in semisolid culture. Moreover, LAA-treated HL-60 showed activity to produce chemiluminescence and expressed CD 66b cell surface antigens, indicating that LAA induces the differentiation of HL-60 mainly into granulocytes. The results are supported by morphological changes of LAA-treated HL-60 into segmented neutrophils. Therefore, the inhibitory effect of LAA on the growth of HL-60 cells seems to arise from the induction of differentiation. To assess the potential role of LAA, cells were exposed to oxygen radical scavengers in the absence or presence of LAA. Catalase abolished and superoxide dismutase promoted LAA-induced differentiation of HL-60. Thus, H_2O_2 produced as a result of LAA treatment seems to play a major role in induction of HL-60 differentiation.

Keywords: L-Ascorbic acid; HL-60; Differentiation; Granulocytes; H_2O_2

INTRODUCTION

L-Ascorbic acid (LAA) is believed to reduce the incidence of a number of so-called "free radical diseases" like cancer and several aging-related pathologies, because in conjunction with other antioxidants, such as vitamin E, ascorbate may reduce oxidative damage.[1,2] On the other hand, ascorbate may also act as an oxidant. In fact, its mode of action depends upon its environment. Moreover, it is believed that, in the presence of molecular oxygen, ascorbate undergoes spontaneous oxidation to form dehydroascorbic acid and the superoxide anion, the latter of which is then reduced to hydrogen peroxide.

However, it has also been reported that pharmacologically attainable concentrations of LAA are toxic to tumor cells *in vitro*.[3–8] In addition, it has been reported that LAA induces apoptotic cell death, which is characterized by cell shrinkage, nuclear fragmentation and internucleosomal DNA cleavage in human myelogenous leukemia cell lines, HL-60, ML-1, and U-937.[9–11] In general, it is believed that the cytotoxic activity of LAA depends on its oxidation-reduction properties. Therefore, it appears that dehydroascorbic acid, the superoxide anion and H_2O_2 may be toxic species and that they are responsible for ascorbate-induced cell death.[12–14]

Several reports have shown recently that leukemia cells are able to differentiate in response to various treatments. Moreover, these reports have suggested that oxygen free radicals may be involved in the mechanism of cell differentiation.[15,16] Thus, it is of importance to determine whether LAA, which generates oxygen free radicals, induces differentiation in addition to apoptosis.

In present study, we used the HL-60 cell line to examine the effect of LAA on the induction of

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leukemia cell differentiation, because it has been reported that this promyelocytic leukemia cell line can be induced to terminally differentiate to morphologically mature granulocytes.[17–19]

MATERIALS AND METHODS

Cell Culture

The human promyelocytic leukemia cell line, HL-60 was obtained from the Korean Cell Line Bank (KCLB). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Exponentially growing cells were used throughout.

Cell Growth Assays

Effect of LAA on the growth of HL-60 cells was determined by the Trypan blue exclusion assay and measurement of metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.[20] One part of 0.16% Trypan blue (Sigma-Aldrich) was added to the cell suspension, and the numbers of unstained (viable) and stained (dead) cells were counted using a hemocytometer. MTT assays were performed as follows. HL-60 cells (3×10^5/ml) were treated for 4 days with various concentrations of LAA (from 10^{-6} to 10^{-3} M) in 96-microwell plates. After incubation, 0.1 mg (50 μl of a 2 mg/ml solution) of MTT (Sigma-Aldrich) was added to each well and cells were then incubated at 37°C for 4 h. Plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. A 150 μl of dimethylsulfoxide was then added to each well to solubilize the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech, UK). All experiments were performed three times and the mean absorbance values were calculated. Results are expressed as the percentage of inhibition that produced reduction of absorbance in LAA-treated cells as compare with untreated controls.

Clonogenic Assay

HL-60 cells (2×10^5/ml) were treated with various concentrations of LAA for 6 days in culture flasks. Harvested cells (2×10^3/ml) were plated in 35 mm petridishes containing methylcellulose-based media (Stem Cell Technologies Inc., Canada), incubated for 12 days, and colonies (≥50 cells) were counted. The results are expressed as the percentage of clonal growth in plates containing LAA-treated cells as compared with the number of colonies in control plate containing cells without treatment.

Assays for Cellular Differentiation

HL-60 cells (2×10^5/ml) were treated with LAA at concentrations varying from 10^{-6} to 10^{-3} M. After incubation for 6 days, cells were harvested and examined for signs of differentiation by analyzing cell surface antigen expression using fluorescence-activated cell sorter (FACS), by observing morphologic changes of Giemsa-stained cells, and by chemiluminescence. To analyze cell surface antigens, cells were stained by direct immunofluorescent staining with fluorescein-isothiocyanate (FITC) conjugated mouse antihuman CD 66b or CD 14, and R-phycoerythrin (PE) conjugated mouse antihuman CD 33. Control studies were performed using non-binding mouse Ig G_1 or Ig M isotype antibodies. The antibodies were purchased from Pharmingen. Fluorescence was measured on a FACStar^{PLUS} flow cytometer (Becton Dickinson, USA). To examine the morphologic changes of HL-60 induced by LAA, cytospin slide was prepared and stained with Wright-Giemsa (Sigma-Aldrich). Chemiluminescence was performed by adding 1.9 ml of Ca^{2+}-free Krebs-Ringer phosphate buffer containing 0.025 mM of luminol (Sigma-Aldrich) and 0.1 ml of cell suspension (1×10^5 cells). After incubating for 5 min at 37°C, formylmethyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich) was added to a final concentration of 10^{-7} M and then light emission was recorded using a luminometer (Berthold, Germany). In order to assess the potential role of LAA in differentiation induction, HL-60 cells (2×10^5/ml) were exposed to catalase (from bovine liver, Sigma-Aldrich) or superoxide dismutase (SOD; from bovine liver, Sigma-Aldrich) (10 U/ml), 15 min before LAA (10^{-3} M) treatment. The expression of cell surface antigens was examined by flow cytometry.

RESULTS

Effects of LAA on HL-60 Proliferation in Liquid Culture and Clonogenicity in Semisolid Culture

The effect of LAA on the growth of HL-60 cells was assessed using a dye exclusion assay and by MTT assay in liquid cultures, and by clonogenic assay in semisolid cultures. Briefly, HL-60 cells were cultured for 4 days in a medium containing various concentrations of LAA. The dye exclusion assay and the MTT assay showed that during a 96 h culture period cell numbers in the control increased almost fourfold, but in the presence of 10^{-3} M LAA these cells no longer proliferated (Table I). The inhibitory

TABLE I Effect of LAA on growth of HL-60 cells in liquid culture

Concentration (M)	Inhibition (%)*	Inhibition (%)†
Control‡	0	0
1×10^{-6}	6.6 ± 0.23	17 ± 3.97*
1×10^{-5}	19 ± 6.12*	24 ± 2.74*
1×10^{-4}	30 ± 0.53*	29 ± 3.42*
1×10^{-3}	61 ± 2.56*	60 ± 1.36*

*$P < 0.05$ compared with the control. *HL-60 cells (2×10^5/ml) were exposed to the indicated concentrations of LAA, and cell viability was measured by trypan blue exclusion after incubating for 96 h. †HL-60 cells (3×10^5/ml) were treated for 4 days with LAA. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubation was continued for 4 h. The formazan salt formed was dissolved in dimethylsulfoxide, and quantified using a microplate reader at 540 nm. The mean absorbance value for each extract was calculated. Results were expressed as the percentage of inhibition that produced reduction of absorbance in extract-treated cells as compare with untreated controls. ‡Without LAA treatment. All experiments were performed in triplicate. Data are presented as means ± SD.

effect of LAA on the growth of HL-60 in liquid culture was significantly greater at concentrations $\geq 10^{-4}$ M. In semisolid cultures, similar clonogenic growth curves were observed (Fig. 1), and treatment of HL-60 cells with LAA (100, 500 and 1000 uM) decreased clonal growth by 49, 60 and 92%, respectively.

Effects of LAA on the Differentiation of HL-60 Cells

HL-60 cell differentiation by LAA was assessed by observing the expression of cell surface antigens. At low concentrations ($< 10^{-4}$ M) of LAA, the ability of HL-60 cells to express CD 66b or CD 14 antigens was unchanged compared with the control. However, the exposure of cells to 10^{-3} M of LAA for 6 days caused the HL-60 cells to express CD 66b or CD 14 antigens (Fig. 2). This expression of CD 66b antigens was marked versus the control, whereas CD 14 antigens expression was only slightly enhanced (Fig. 3). These results show that HL-60 cells are differentiated to mature granulocytes or monocytes by LAA, and are supported by striking changes in HL-60 morphology, which are characteristic of mature granulocytes (metamyelocytes, banded and segmented neutrophils), induced by LAA (Fig. 4). In differentiated myeloid cells, chemotatic peptide fMLP is capable of inducing luminol-dependent chemiluminescence response.[21] Neutrophilic differentiation of HL-60 cells could also be verified by chemiluminescence related to the respiratory burst activation in polymorphonuclear leucocytes.[22] So, whether LAA-treated cells produce chemiluminescence or not, was observed. Exposure of cells to LAA ($\geq 10^{-4}$ M) for 6 days resulted in HL-60 cells producing chemiluminescence (Fig. 5).

Effects of Radical Scavengers on the Differentiation of HL-60

HL-60 cells were exposed to oxygen radical scavengers in the absence or presence of LAA. The expression of cell surface antigens was examined by flow cytometry (Figs. 6 and 7). Catalase (10 U/ml) abolished and SOD (10 U/ml)

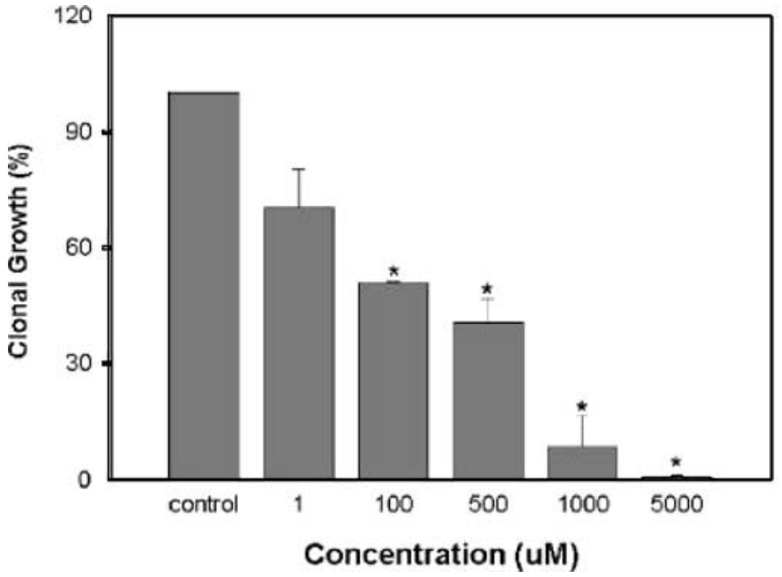

FIGURE 1 Effect of LAA on the clonogenicity of HL-60. HL-60 (2×10^5/ml) were incubated with various concentrations of LAA in liquid culture for 6 days, and LAA-treated-HL-60 cells (2×10^3/ml) were also cultured in semisolid culture. Colonies (≥50 cells) were counted after 12 days of incubation. Results are expressed as the percentage of clonal growth. Data represent the means ± SEM of three experiments. Relative to no LAA treatment taken as 100%. *$P < 0.05$ compared with the control.

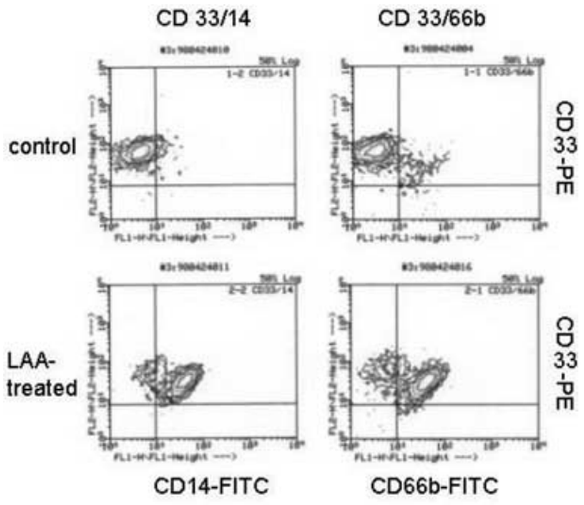

FIGURE 2 Differentiation of HL-60 cells as induced by LAA. Cells were treated for 6 days with LAA (10^{-3} M) and the expressions of CD 66b and CD 14 antigens were analyzed by FACS.

promoted the LAA-induced differentiation of HL-60 cells (Table II), indicating that the effect of LAA on HL-60 differentiation is diminished by catalase and potentiated by SOD. This suggests a pivotal role for H_2O_2 in differentiation induced by LAA, and that differentiation induction by LAA might be initiated by oxygen free radical mediated reaction.

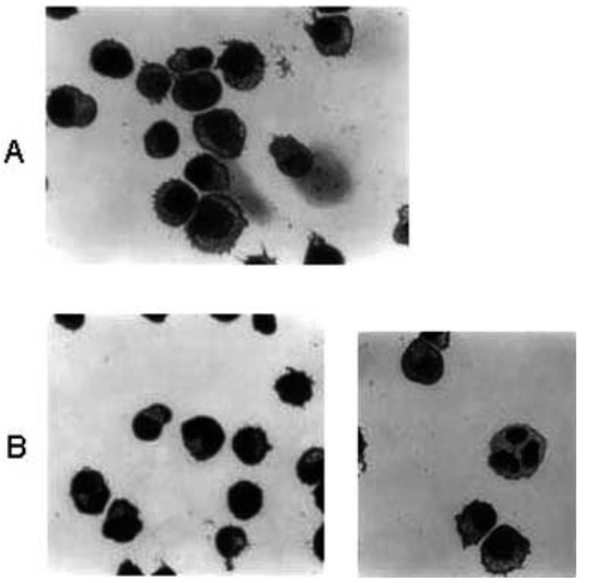

FIGURE 4 Morphology of LAA-treated HL-60 cells. Cytospin slide preparation of suspension cell cultures stained with Wright-Giemsa (× 400). (A) Cells cultivated without LAA consisted of promyelocytes with characteristic cytoplasmic granules, large nuclei and prominent nucleoli. (B) Cells cultured in 10^{-4} M LAA for 6 days consisted of metamyelocytes and segmented neutrophils.

DISCUSSION

It is believed that LAA is cytotoxic to some tumor cells *in vitro* and that its effect depends on its oxidation–reduction properties.[9–11] Also, several

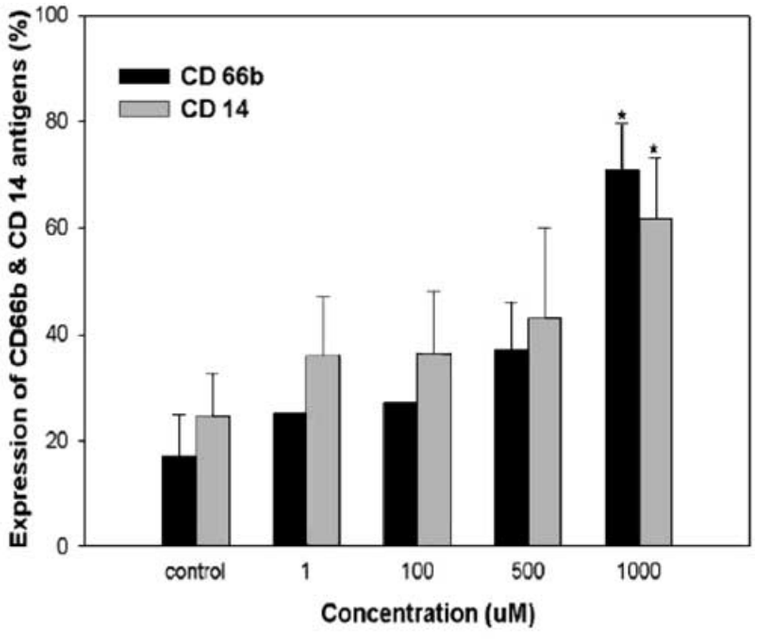

FIGURE 3 Effect of LAA on HL-60 differentiation. Cells were seeded at 2×10^5/ml. Differentiation was determined by examining the expressions of CD 66b and CD 14 antigens after 6 days of growth in the various concentrations of LAA. Data represent the means ± SEM of three experiments. *$P < 0.05$ compared with the control.

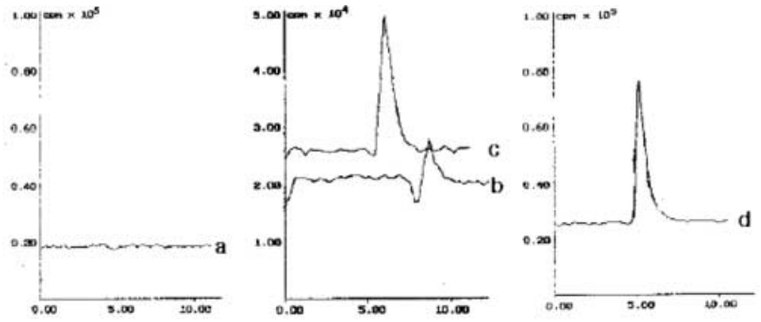

FIGURE 5 Time trace of the chemiluminescence produced by LAA-treated HL-60 cells in the presence of luminol (2.5×10^{-5} M), when exposed to formylmethyl-leucyl-phenylalanine (10^{-7} M). Abscissa: Time of study (minutes). Ordinate: Chemiluminescence in counts per minute (cpm) (a and d; cpm $\times 10^5$, b and c; cpm $\times 10^4$). (a) Cells without LAA treatment; (b) 100 uM LAA-treated cells; (c) 500 uM LAA-treated cells; (d) 1000 uM LAA-treated cells.

reports have suggested that oxygen free radicals may be involved in cell differentiation.[15,16] In this study, we investigated the effect of LAA on the differentiation induction of HL-60 which can be induced to terminally differentiate to morphologically mature granulocytes.[17–19] To our knowledge, this study shows for the first time that LAA induces the differentiation of HL-60 and the H_2O_2 produced from LAA possibly plays a major role in differentiation.

We observed that LAA inhibited the cellular and clonal growths of HL-60 cells, suggesting that LAA induced differentiation and/or apoptosis. In present study, we observed that LAA induced the differentiation of HL-60 mainly into granulocytes. Also, we observed the induction of apoptosis by flow cytometry. When HL-60 cells were stained with the DNA-specific fluorochrome, propidium iodide, LAA was found to increase the proportion of sub-G1 hypodiploid cells (not shown). Many studies have been performed on the induction of differentiation and apoptosis in HL-60 cells. HL-60 cells were induced to differentiate into granulocyte-like cells by dimethyl sulfoxide[18] and retinoic acid,[17,23] or into monocyte(macrophage)-like cells by 1,25-dihydroxyvitamin D_3[24] and phorbol diesters.[25–27] On the other hand, it was reported that ascorbate or parthenolide increases the 1,25-dihydroxyvitamin D_3-induced monocytic differentiation of HL-60 cells.[28,29] It has also been reported that ascorbates, gallates or benzo(a)phenothiazines induce apoptosis.[12,30,31]

When several leukemic cell lines, such as HL-60, U937, and K562, were cultured with various compounds, they were differentiated to phagocytic-like cells that can produce O_2^-. During differentiation, protein components essential for O_2^- generation are thought to be induced in these cells.[32–34] Moreover, non-differentiated cells and tumor cells usually have lower SOD activities than

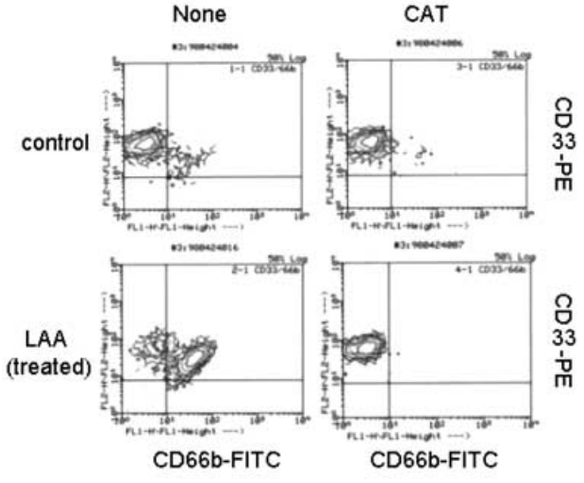

FIGURE 6 Expression of CD 33 and CD 66b antigens on HL-60 cells. HL-60 cells (2×10^5/ml) were exposed to catalase (CAT: 10 U/ml) in the presence or absence of LAA (10^{-3} M) for 6 days, and then analyzed by FACS.

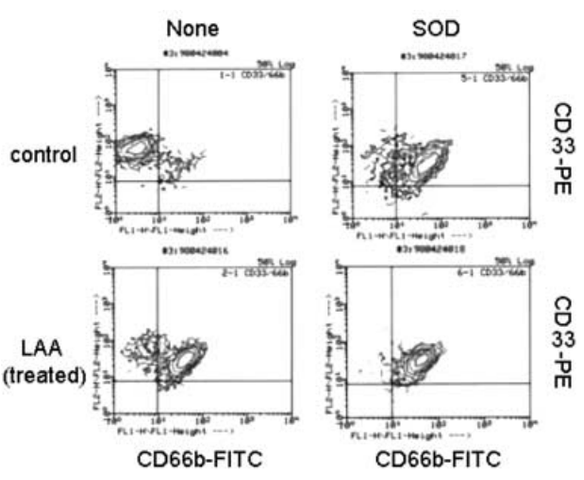

FIGURE 7 Expression of CD 33 and CD 66b antigens on HL-60 cells. HL-60 cells (2×10^5/ml) were exposed to superoxide dismutase (SOD: 10 U/ml) in the presence or absence of LAA (10^{-3} M) for 6 days, and then analyzed by FACS.

TABLE II Effect of oxygen radical scavengers on differentiation of HL- 60 cells by LAA

Oxygen radical scavengers	LAA (M)	Expression of CD 66b (%)	Expression of CD 14 (%)
None	Control*	3	4.5
	10^{-3}	87	92
CAT (10 U/ml)	Control*	1	5.6
	10^{-3}	1	10
SOD (10 U/ml)	Control*	94	97.9
	10^{-3}	98	98

*Without LAA treatment. HL-60 cells (2×10^5 cells/ml) were exposed to oxygen radical scavengers (catalase: CAT or superoxide dismutase:SOD) in the presence or absence of LAA for 6 days, and then analyzed by FACS.

differentiated cells. Furthermore, SOD activity increases dramatically in a variety of tumor cell systems when they undergo differentiation.[35–37] These reports show that a transient increase in SOD activity may be a prerequisite for the onset of cell maturation, and support our data, which show that SOD treatment induces the differentiation of HL-60 cells. On the other hand, CAT inhibited the differentiating effect of LAA on HL-60 cells. Therefore, the H_2O_2 produced by SOD or LAA possibly plays a major role in the induction of leukemia cellular differentiation.

It is important for understanding how LAA induces the differentiation. Candidate molecules for mediating the differentiation of HL-60 are retinoic acid receptors, myc and myeloblastin. Therefore, we are scheduled to do further studies for expression of these molecules.[38–41]

Acknowledgements

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