Effect of DL- α -Tocopherol (Vitamin E) on the Differentiation of Mouse Myeloid Leukemia Cell

Hiroshi SAKAGAMI,¹ Kazumi ASAKA,¹ Etsuko ABE,²
Chisato MIYAURA,² Tatsuo SUDA,²
and Kunio KONNO¹

¹*Department of Biochemistry, School of Medicine,* ²*Department of Biochemistry,
School of Dentistry, Showa University,
1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan*

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Summary The effect of DL- α -tocopherol on the differentiation of the mouse myeloid leukemia cell line (M1) was investigated using fluorometry. The reliability of the fluorometric determination was verified by the mutual overlapping of the curves of disappearance of the radioactivity recovered as α -tocopherol and the loss of its fluorescence. α -Tocopherol solubilized into the culture medium was relatively stable ($t_{1/2}$ = 147–168 hr). Incorporation of α -tocopherol into M1 cells was time- and dose-dependent. Proliferation of M1 cells was never inhibited, but was slightly enhanced by treatment with α -tocopherol. α -Tocopherol caused a twofold increase in acid-phosphatase activity and slightly inhibited both the spontaneous- and dexamethasone-induced differentiation with respect to adhesion on glass and cell surface-rosette formation. α -Tocopherol also induced morphological change, making the shape of M1 cells rounder.

Key Words α -tocopherol, vitamin E, stability, differentiation, adhesion, acid-phosphatase, rosette formation

It is generally accepted that DL- α -tocopherol (vitamin E) is an antioxidant and a radical scavenger in various metabolic processes (1, 2) and stabilizes cell surface membranes and the membranes of subcellular organelles by its physicochemical interactions (3, 4). Tissue culture technique has been used by some investigators to study the function of α -tocopherol (5–7). However, these authors have explained their findings only in terms of the above-mentioned functions of α -tocopherol. Considering the possibility that α -tocopherol may play some other physiological

¹ 坂上 宏, 浅香一美, ² 阿部悦子, 宮浦千里, 須田立雄, ¹ 紺野邦夫

All inquiries should be addressed to Hiroshi Sakagami, Department of Biochemistry, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

roles, we initiated a series of experiments to study the role of α -tocopherol in cellular aging (8) and cell differentiation.

Here, we report our findings on the effect of α -tocopherol on cell differentiation.

MATERIALS AND METHODS

Cell culture. The cells used in this experiment are mouse myeloid leukemia cells (M1) (clone T22), which are known to differentiate into granulocytes and macrophages when treated with various inducers (9, 10). They were cultured in suspension in Eagle's minimal essential medium (MEM) which contained twice the normal concentrations of amino acids and vitamins, and which was supplemented with 10% heat-inactivated (56°C, 30 min) calf serum (Chiba Serum Institute, Japan).

Reagents. DL- α -Tocopherol (vitamin E) (Eisai Co., Ltd.) and DL- α -[5-methyl-³H]tocopherol (1 Ci/mmol, Radiochemical Centre, Amersham) were solubilized as described below. Dexamethasone sodium phosphate (Orgadron) (Sankyo Co., Ltd.) was dissolved in a phosphate-buffered saline lacking calcium and magnesium (PBS(-)).

Quantitative determination of α -tocopherol. The concentration of α -tocopherol in culture medium was routinely estimated by fluorometric determination, utilizing the fluorescence of the chroman ring in the α -tocopherol molecule (11).

Method of solubilizing α -tocopherol into the culture medium. An oil droplet of α -tocopherol was solubilized by mixing into heat-inactivated calf-serum to avoid the use of organic solvents. After mixing with 9 vol. of MEM containing twice the normal concentrations of amino acids and vitamins, the micelles of α -tocopherol were removed by millipore filtration as described previously (8).

Collection of adherent cells. The supernatant cells of cultures treated with α -tocopherol or dexamethasone were pooled, and the loosely adherent cells were collected by gently rinsing three times with pre-warmed culture medium (37°C). These cells were pooled and designated nonadherent cells. The cells that still adhered after rinsing with the culture medium were designated adherent cells and collected by pipetting with cold PBS(-) (0°C).

Enzyme assay. The lysozyme activity was assayed as described previously (12). In brief, cells were suspended in distilled water, at a density of 4×10^7 cells/ml, and disrupted at 0°C for 5 min by sonication. The cell lysate (20 μ l) was applied to a small well (ϕ 4 mm) made on the lysoplate which was solidified with 1% (w/v) Difco agar containing 0.5% (w/v) heat-killed *Micrococcus Lysodeikticus* (Sigma) and 50 mM NaCl 67 mM sodium phosphate at pH 5.9. After the plates had been incubated for 24 hr at 37°C, the diameters of the cleared zones on the plates were measured. One unit of activity was defined as the amount equivalent to 1 μ g of egg white lysozyme (Seikagaku Kogyo Co.) under the same conditions.

The acid-phosphatase activity was assayed by a modification of the method described by Mitchell *et al.* (13). First, 0.1 ml of cell lysate (0.5×10^7 cells/ml of

distilled water) was mixed with 1 ml of 38 mM sodium citrate buffer (pH 4.8), 0.1% Triton X-100 and 4.2 mM *p*-nitrophenyl phosphate (Sigma Chemical Co.). After incubation for 15 min at 37°C, the reaction was stopped by the addition of 2 ml of 0.1 M NaOH. The optical density of the *p*-nitrophenol was determined at 410 nm and the acid-phosphatase activity was calculated from the calibration curve for commercial *p*-nitrophenol.

The β -glucuronidase activity was assayed by a modification of the method described by Mitchell *et al.* (13). Cell lysate (0.5×10^7 cells/ml of distilled water; 0.1 ml) was mixed with 1 ml of 0.1 M sodium acetate buffer (pH 4.5), 0.1% Triton X-100 and 2.7 mM *p*-nitrophenyl- β -D-glucuronide (Sigma Chemical Co.). The enzymic hydrolysis of the substrate was achieved by incubation at 37°C for 1 hr. The reaction was stopped by the addition of 2 ml of 0.5 M NaOH and the optical density of the *p*-nitrophenol was determined at 410 nm to assess the enzyme activity.

Assay for surface receptors for immunoglobulin and immunoglobulin complement. This assay was based on rosette formation by sheep erythrocytes coated with antibody (EA) or with antibody and complement (EAC). Rosette formation was tested by the method described by Bianco *et al.* (14). The EA or EAC cells were washed three times with PBS(–) and resuspended at 10^9 cells/ml in serum-free culture medium. To quantify the percentage of cells with a rosette, 5×10^7 EA or EAC cells were mixed with 10^6 M1 cells in a volume of 1 ml, centrifuged and incubated for 30 min at 37°C. The pellet was dispersed and the percentage of cells with 4 or more attached erythrocytes was determined in a hemocytometer by counting about 200 cells.

Separation of α -tocopherol from tocopherylquinone. Culture medium (0.5 ml) containing [3 H]tocopherol and its degradation product during incubation, was mixed well with 0.5 ml of ethyl alcohol, then with 2.5 ml of *n*-hexane. After centrifugation, the *n*-hexane phase was collected and evaporated off. The residue was dissolved in 100 μ l of *n*-hexane and 20 μ l aliquots were then applied to Silica gel G plate (Pre-coated TLC Plates, Merck) and developed with a mixed solvent of ethylether: benzene (2:8). After the solvent front nearly reached the top, the regions to which authentic α -tocopherol ($R_f=0.86$) and tocopherylquinone ($R_f=0.57$) migrated were scraped off the plates. The radioactivities were extracted twice with methanol, evaporated under vacuum, solubilized with 5 ml of toluene scintillator, and then assayed with a liquid scintillation spectrometer. The average recovery of the radioactivity after thin layer chromatography was $99.3 \pm 3.7\%$ ($n=7$).

Chemical determination. The amount of protein was determined by the method of Lowry *et al.* (15) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Solubilization of α -tocopherol into culture medium

The calf serum used in the following experiments (Chiba Serum Institute, Japan) contained less than $0.4 \mu\text{M}$ of α -tocopherol. This is only 2% of the α -

Fig. 1. Degradation of α -tocopherol in culture medium during incubation. Authentic or radioactive α -tocopherol was solubilized into culture medium as described in MATERIALS AND METHODS. To facilitate the degradation of α -tocopherol, the composition of the culture medium was modified as follows: MEM supplemented with 10% (v/v) calf serum (Flow Laboratories, U.S.A.) (See Table 1). The relative fluorescence intensity remaining after incubation for the indicated times in the CO_2 -incubator (O) was extracted and determined by fluorometric determination (11). The relative remaining radioactivity, which migrated to the position of authentic α -tocopherol on the TLC plates (Merck) after incubation (●), was determined as described in MATERIALS AND METHODS.

tocopherol level in normal human serum ($20 \mu\text{M}$) (8). For accurate assessment of the function of α -tocopherol, we previously explored a method of solubilizing α -tocopherol (8). When α -tocopherol is solubilized into culture medium, it closely resembles its physiological state in normal human serum, with respect to the lack of micelles and the distribution pattern in the lipoprotein fraction (8). First, we investigated the reliability of fluorometric determination of α -tocopherol. Figure 1 shows that α -tocopherol is degraded during incubation in culture medium and that the logarithms of both the remaining fluorescence of α -tocopherol and the radioactivity recovered from the region of authentic α -tocopherol on TLC plate yielded straight lines against the incubation time, the slopes of which were almost indistinguishable from each other. This indicates that the amount of undegraded α -tocopherol can be accurately determined by fluorometric determination. The half-life of α -tocopherol solubilized into the presently used culture medium is much longer than the value we had previously reported ($t_{1/2} = 32 \text{ hr}$, see Ref. 8) (Table 1). Table 1 shows that this stabilization of α -tocopherol in the present system was not due to the effect of heat-inactivation of the serum, nor to the presence of the cells in the culture medium, but rather to the difference in the components of these two sera. The serum components which stabilize α -tocopherol remain to be determined.

Incorporation of α -tocopherol into M1 cells

As shown in Fig. 2A, the amount of α -tocopherol incorporated in a given number of M1 cells increased up to 20 hr after treatment, maintained a constant

Table 1. Stability of α -tocopherol.

Medium	Presence of cells in the medium	Half-life of α -tocopherol (hr)	No. of experiments
MEM ^a + 10% heat-inactivated calf serum ^b	+	155 $\pm$ 51 ^d	6
MEM ^a + 10% heat-inactivated calf serum ^b	—	168 $\pm$ 52	3
MEM ^a + 10% calf serum ^b	—	147 $\pm$ 48	3
MEM + 10% calf serum ^c	—	32 $\pm$ 1	3

^a MEM with twice the normal concentrations of amino acids and vitamins. ^b Purchased from Chiba Serum Institute, Japan. ^c Purchased from Flow Laboratories, U.S.A.

^d Mean $\pm$ SE.

Fig. 2. Kinetics of α -tocopherol incorporation into M1 cells. (A) Time course. M1 cells (2×10^6 cells) were incubated for the indicated times with 10 ml of 25 μ M α -tocopherol. The cells were then harvested, washed three times with PBS(—) and disrupted by suspension in cold distilled water. α -Tocopherol incorporated into the cells was extracted and determined as described previously (8). (B) Dose-response. M1 cells (2×10^6 cells) were incubated for 42 hr with 10 ml of the indicated concentrations of α -tocopherol. The amount of α -tocopherol incorporated into the cells was then determined as described in (A).

value up to 60 hr, and thereafter slightly decreased. The fluorescence intensity of α -tocopherol (excitation 300 nm, emission 320 nm), released from the untreated M1 cell lysate, was negligible. As shown in Fig. 2B, α -tocopherol incorporation was dose-dependent. About 1–2% of α -tocopherol initially added to the culture medium was incorporated into M1 cells after 42-hr treatment.

Fig. 3. Effect of α -tocopherol on M1 cell proliferation. Each point represents a doubling time, calculated from the growth curve of exponentially growing M1 cells which were treated with the indicated concentrations of α -tocopherol.

Fig. 4. Effect of α -tocopherol on M1 cell adhesion on glass surface. (A) M1 cells were incubated for the indicated times with either 0 (●), or 8 (□) or 80 μ M (△) of α -tocopherol. The adherent cells were then collected as described in MATERIALS AND METHODS. (B) M1 cells were incubated with either 0 (●), or 8 (□), or 80 μ M (△) of α -tocopherol in the presence of 1 μ M dexamethasone.

Effect of α -tocopherol on M1 cell proliferation

Since the doubling time of exponentially growing M1 cells was slightly shortened with increased concentration of α -tocopherol in the culture medium ($\sim 70 \mu$ M), the proliferation of M1 cells seemed to be slightly enhanced by treatment with α -tocopherol (Fig. 3).

Effect of α -tocopherol on M1 cell adhesion on glass surface

As shown in Fig. 4A, control M1 cells spontaneously produced a cell population that adhered to glass surfaces (20% of total cells). The addition of α -tocopherol (8 or 80 μ M) to this culture caused a 40% decrease in the formation of

Table 2. Effect of α -tocopherol on the formation of EA and EAC receptors. M1 cells were treated for 72 hr with the indicated concentrations of α -tocopherol ($\pm 1 \mu\text{M}$ dexamethasone) and the formation of EA or EAC rosettes was assayed as described in MATERIALS AND METHODS.

Treatment	% cells with rosette	
	EA receptor	EAC receptor
Control	6.9 (100)	7.6 (100)
8 μM α -tocopherol	3.0 (43)	4.7 (61)
80 μM α -tocopherol	6.7 (97)	4.1 (54)
1 μM dexamethasone	16.5 (238)	24.3 (319)
1 μM dexamethasone + 8 μM α -tocopherol	20.9 (300)	20.4 (268)
1 μM dexamethasone + 80 μM α -tocopherol	11.1 (160)	19.6 (257)

adherent cells. Since the previous studies have shown that dexamethasone makes M1 cells adhere to glass surfaces (60–70% of total cells at the maximum) (12, 16), we investigated whether α -tocopherol competes with the action of dexamethasone. As shown in Fig. 4B, α -tocopherol slightly inhibited (9–25%) the production of adherent cells caused by treatment with 1 μM dexamethasone. This inhibitory effect of α -tocopherol was reproduced in two other independent experiments (data not shown).

Effect of α -tocopherol on the formation of EA and EAC receptors

The induction of cell surface receptors for immunoglobulin and immunoglobulin complement is also a good marker for M1 cell differentiation (17). Table 2 shows that control M1 cells spontaneously produce a cell population that forms EA and EAC receptors in cell surface membrane (7–8% of total cells) and that, as in previous studies (17), the dexamethasone treatment caused a two to threefold increase in the formation of these receptors. On the other hand, the addition of α -tocopherol (8 or 80 μM) reduced the number of these cell surface receptors to 43–97% of the control value (Table 2). Large amounts of α -tocopherol (80 μM) also appeared to slightly inhibit the dexamethasone-induced enhancement of the receptor formation.

Effect of α -tocopherol on induction of lysosomal enzymes

Dexamethasone induced and/or enhanced lysozyme, β -glucuronidase and acid-phosphatase activities, in agreement with the result of Nagata *et al.* (18), whereas α -tocopherol increased only acid-phosphatase activity among these enzymes (Table 3). The activation of acid-phosphatase by α -tocopherol, at concentrations above 4 μM , was slightly superior to that of dexamethasone. This was confirmed by repeated experiments in which treatment for 72 hr with 25 μM α -tocopherol or 1 μM dexamethasone increased the acid-phosphatase activity over that of the control by

Table 3. Effect of α -tocopherol on the induction of lysosomal enzymes.

M1 cells were incubated for 72 hr with the indicated concentrations of α -tocopherol or dexamethasone, and then lysosomal enzyme activities were assayed as described in MATERIALS AND METHODS.

Treatment	Lysozyme activity (unit/mg protein)	β -Glucuronidase activity (nmol <i>p</i> -nitrophenol released/min /mg protein)	Acid-phosphatase activity (nmol <i>p</i> -nitrophenol released/min /mg protein)
Control	0.48 (100) ^a	6.0 (100) ^a	11.3 (100) ^a
0.2 μ M α -tocopherol	0.48 (100)	—	—
2	0.49 (102)	6.3 (105)	—
4	—	—	16.0 (142)
8	—	—	22.2 (197)
20	0.48 (100)	5.3 (89)	—
40	—	—	20.6 (182)
80	—	—	26.7 (236)
1 μ M dexamethasone	3.55 (740)	11.1 (187)	18.2 (161)

^a % of control.

174 $\pm$ 24% and 151 $\pm$ 17% ($n=3$) respectively.

Studies of α -tocopherol on cell differentiation have been described in much less detail (19). In this experiment, we investigated the effect of α -tocopherol on the differentiation of the mouse myeloid leukemia cell line (M1). We found that various physiological concentrations of α -tocopherol (2–80 μ M) slightly inhibited both the spontaneous and dexamethasone-induced differentiation, with respect to adhesion (Fig. 4) and cell surface-rosette formation (Table 2). However, this effect of α -tocopherol was not so strong as to return the percentage of the differentiated M1 cells to the original level (Table 2 and Fig. 4B). α -Tocopherol also induced morphological change, making the shape of M1 cells rounder (data not shown). This morphological change may be associated with the alteration in cell surface membrane, possibly caused by the hydrophobic interaction of α -tocopherol with some membrane components (3, 4).

It seems unlikely that the effects of α -tocopherol described here are due either to its degradation during culture, or its nonspecific binding to cell surface membrane, since α -tocopherol solubilized into culture medium is relatively stable (Table 1), is incorporated into M1 cells in a time-dependent manner (Fig. 2) and selectively activates only acid-phosphatase among three lysosomal enzymes (Table 3).

The relationship between α -tocopherol-mediated slight inhibition of M1 cell differentiation and enhancement of acid-phosphatase remains to be investigated.

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