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Altered production of the active oxygen species is involved in enhanced cytotoxic action of acylated derivatives of ascorbate to tumor cells

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Our previous study shows that 6-*O*-acyl derivatives of L-ascorbic acid inhibits more markedly cell growth of mouse Ehrlich carcinoma than ascorbic acid. The present study shows that 6-*O*-palmitoyl ascorbic acid but not ascorbic acid prolongs the lifespan of mice into which tumors such as Meth A fibrosarcoma, MM46 mammary carcinoma, Ehrlich carcinoma and sarcoma 180 are implanted. The potenciated cytotoxicity of 6-*O*-palmitoyl ascorbic acid is not due to an increase in duration time of the cytotoxic action, because 6-*O*-palmitoyl ascorbic acid is gradually inactivated during contact with tumor cells and exhibits a similar action time curve to that of ascorbic acid as shown by clonal growth assay. Cytotoxicity of 6-*O*-palmitoyl ascorbic acid is markedly diminished by combined addition of catalase and superoxide dismutase (SOD), as shown by dye exclusion assay, whereas the cytotoxicity was slightly reduced by either enzyme alone but not by the specifically inactivated or heat-denatured enzymes. In contrast, cytotoxicity of ascorbic acid is abolished by catalyse but not SOD. Autooxidation of 6-*O*-palmitoyl ascorbic acid was not inhibited by catalase plus SOD. The results indicate that cytotoxicity of 6-*O*-palmitoyl ascorbic acid is attributed at least partly to both hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) generated at the early stage. Cytotoxicity of 6-*O*-palmitoyl ascorbic acid is also appreciably attenuated by singlet oxygen ($^1O_2$) scavengers such as hydroquinone, 1,4-diazobicyclo-2,2,2-octane or sodium azide, but not by hydroxyl radical scavengers including butylated hydroxytoluene, D-mannitol, benzoic acid and ethanol. Thus, in contrast to cytotoxicity of ascorbic acid mediated entirely by H_2O_2 initially generated, acylated ascorbic acid produces a diversity of active oxygen species including H_2O_2, $O_2^{\cdot-}$ and other species secondarily generated via disproportion, which may be additively involved in the enhanced cytotoxic action.

Introduction

Ascorbic acid and its oxidized form dehydroascorbic acid are postulated to play an important role in inhibitory control to cell growth and division in the animal tissue [1]. Ascorbic acid has been reported to exhibit a cytotoxic activity at

Abbreviations: SOD, superoxide dismutase (EC 1.15.1.1); MEM, Eagle's minimum essential medium; FCS, fetal calf serum; PSS, physiological salt solution.

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high concentrations or in combination with supplements [2–11].

We found that ascorbic acid in chelation with a ferrous ion enhances the cell growth-inhibiting activity through prolonged duration of the cytotoxic action [12]. In our previous study [13] 6-*O*-acylated derivatives of ascorbic acid such as 6-*O*-palmitoyl and 6-*O*-stearoyl ascorbic acid are shown to inhibit cell growth of Ehrlich ascites tumor. In contrast, 2,6-dipalmitoyl ascorbic acid or an equimolar mixture of ascorbic acid and palmitic acid, stearic acid or their methyl esters is not cytotoxic, suggesting that the enhanced cytotoxicity is primarily due to balanced hydrophobicity introduced into an ascorbic acid molecule by a poorly cytotoxic acyl moiety [13].

The present study demonstrates that more potent growth-inhibition of 6-*O*-palmitoyl ascorbic acid than ascorbic acid is attributed to altered production of the active oxygen species but not to change in duration of the growth-inhibiting action.

Materials and Methods

Chemicals

6-*O*-Palmitoyl ascorbic acid (Tokyo Chem. Industry) in PSS or MEM (Nissui Pharm., Tokyo)/10% FCS (Armour Pharm.) supplemented with 0.5–1.0% Pluronic F68 (Asahi Denka, Tokyo) was emulsified with a Potter-Elvehjem Teflon homogenizer. Ascorbic acid (Wako Chem., Osaka) was dissolved similarly. The water was prepared with a Milli-RO 8/Milli-Q reagent water system.

Treatment of tumor-implanted mice

Tumor cells such as MM46 mammary carcinoma, Meth A fibrosarcoma, Ehrlich carcinoma, sarcoma 180 (described above, $2 \cdot 10^6$ cells), P388 ($1 \cdot 10^6$) and L1210 ($5 \cdot 10^5$) leukemia, P815 mastocytoma ($1 \cdot 10^6$) and B16 melanoma ($1 \cdot 10^6$) were inoculated i.p. into mice (25 g) such as C3H/He, Balb/c, ddY, ICR, CDF_1, DBA/2, DBA/2 and C57BL/6, respectively. The mice were obtained from Shizuoka Agric. Co-op. Association for Laboratory Animals, Hamamatsu, Japan. The mice were treated i.p. daily with 0.25 ml of drug emulsion for 5 successive days. The control mice received 1.0% Pluronic F68 in PSS simmilarly.

Heat-denaturation and inactivation of catalase and SOD

Catalase from bovine liver (EC 1.11.1.6, thymol-free, $1.0–2.5 \cdot 10^4$ units/mg protein, Sigma Chemicals) or recombinant human Cu/Zn-SOD (EC 1.15.1.1., 4450 units/mg protein, Ube Kohsan, Yamaguchi, Japan) solution (600 unit/ml) was heated at 100 °C for 5 min. SOD was inactivated by exposure to 5 mM H_2O_2 (Wako Chemicals, Osaka) at pH 9.5 for 1 h at 25 °C followed by dialysis against 0.1 M sodium phosphate buffer (pH 7.2). Catalase was inactivated with 0.1 M 3-amino-1,2,4-triazole (Sigma chemicals) and 5 mM H_2O_2 in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 8 h; then it was repeatedly dialyzed for 7 days against the buffer containing no other agents essentially as described by Margoliash et al. [15].

Clonal growth assay

Colony formation of human lung carcinoma A549 cells was carried out as previously described [14]. The cells at the exponential growth phase were trypsinized. Suspension of the monodispersed cells ($1.0 \cdot 10^2–2.5 \cdot 10^3$ cells per 0.1 ml) was inoculated on a 60 mm Nunclon plastic dish containing 3.9 ml of MEM/10% FCS. After incubation at 37 °C in 5% CO_2/air for 42 h, 0.2 ml of 6-*O*-palmitoyl ascorbic acid emulsion was added. At the indicated treatment times, the medium was drained and substituted with MEM/10% FCS with or without 6-*O*-palmitoyl ascorbic acid of the same concentration which was freshly prepared. After 13 days of treatment, the colonies formed were fixed with formalin and stained with Crystal violet. Colonies composed of more than 50 cells were counted. The ratio of colony number of a drug-treated dish versus that for the control dish similarly treated without drug was determined and is designated as a cloning efficiency (%).

Dye exclusion assay

A549 cells ($2 \cdot 10^5$) suspended in 1.0 ml of MEM/10% FCS were seeded on 16-mm wells of a Costar microplate. After 24 h incubation, 0.1 ml of catalase or SOD solution (600 unit/ml), and then 0.2 ml of medium containing ascorbic acid or 6-*O*-palmitoyl ascorbic acid were added. After 48 h treatment, the cells were feeded with 0.17%

Trypan blue in PSS. The proportion of the number of dye-excluding cells to the total was determined. The ratio of the proportion in a drug-treated well versus that in a control well treated without drug is denominated as a survival cell fraction.

Cell growth assay

Mouse Ehrlich ascites tumor cells or mouse neuroblastoma NAs-1 cells ($1 \cdot 10^3$ per 0.1 ml) were seeded in 16-mm wells of a Costar microplate containing 0.9 ml of MEM or Dulbecco's modified MEM/10% FCS, respectively. After 24 h incubation, 0.2 ml of medium containing catalyse, SOD or other scavengers (Wako Chem.) and then 0.2 ml of medium containing ascorbic acid or 6-*O*-palmitoyl ascorbic acid were added. The control cells received the medium containing the scavenger but not ascorbic acid or 6-*O*-palmitoyl ascorbic acid. After 5 days of treatment, when the control cells still grew exponentially, cell numbers were determined with a Coulter electric corpuscle counter ZBII.

Autooxidation of ascorbic acid and 6-O-palmitoyl ascorbic acid

3 ml of MEM/10% FCS containing 80 μM ascorbic acid or 6-*O*-palmitoyl ascorbic acid in a Nunclon 60-mm plastic dish was retained at 37 °C and pH 7.2 in 5% CO_2/air. The unoxidated compound was quantified by iodine-starch method, which shows no differences as compared with hydrazine method and indophenol method [17]. The fluid containing ascorbic acid or 6-*O*-palmitoyl ascorbic acid was washed out with 40 ml of 2% metaphosphate. After addition of 3 ml of 0.5% starch as an indicator, it was titrated with 0.1 or 0.01 M iodine. The coexistent materials did not disturb the calibration of unoxidated compound.

Results

Prolonging effects of 6-O-palmitoyl ascorbic acid on survival time of tumor-implanted mice

6-*O*-Palmitoyl ascorbic acid was intraperitoneally administered to mice into which a variety of tumors had been intraperitoneally implanted. 6-*O*-palmitoyl ascorbic acid exerted repressive effects on tumors such as MM46 mammary carcinoma, Meth A fibrosarcoma, Ehrlich carcinoma and sarcoma 180 in a dose-dependent manner (Fig. 1). Doses of 25–50 mg 6-*O*-palmitoyl ascorbic acid per kg gave the maximal lifespans of tumor-implanted mice, which were 1.9–3.3-times as long as those of the untreated mice. No increase in the lifespans was observed even at a dose of 100 mg 6-*O*-palmitoyl ascorbic acid per kg for tumors such as P815 mastocytoma, B16 melanoma, P388 and L1210 leukemias. In contrast, ascorbic acid did not prolong the lifespan even at doses as high as 100–200 mg/kg for any type of tumor.

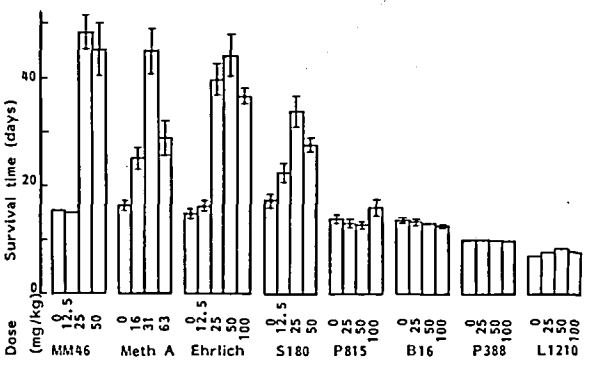

Fig. 1. Effects of 6-*O*-palmitoyl ascorbic acid on the lifespans of tumor-implanted mice. 6-*O*-palmitoyl ascorbic acid emulsified with Pluronic F68 in PSS was i.p. administered for 5 successive days into mice into which a variety of tumor cells had been implanted: MM46 mammary carcinoma, Meth A fibrosarcoma, Ehrlich carcinoma, sarcoma 180, P815 mastocytoma, B16 melanoma, P388 and L1210 leukemias. The control mice received Pluronic F68 solution in PSS. The bar represents S.E. for 6–10 mice used for each experimental group.

Duration of cytotoxic action of 6-O-palmitoyl ascorbic acid during contact with tumor cells

6-*O*-palmitoyl ascorbic acid was administered to monodispersed cells of human lung carcinoma A549. At drug-treatment times indicated, the medium was replaced by fresh medium with or without 6-*O*-palmitoyl ascorbic acid. Therefore the experiment shows the difference in cytotoxic effects of duplicate administration and single shot of 6-*O*-palmitoyl ascorbic acid. The colonies formed were counted, and dependence of 6-*O*-palmitoyl ascorbic acid cytotoxicity on the action time was examined by clonal growth assay (Fig. 2). 6-*O*-palmitoyl ascorbic acid was gradually in-

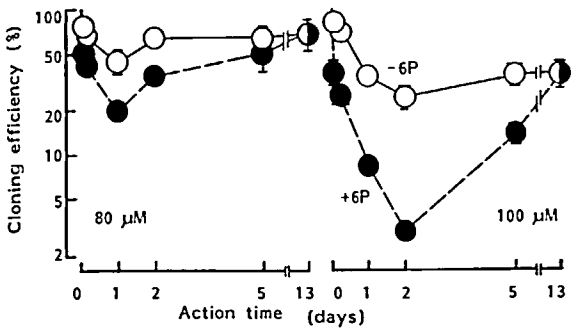

Fig. 2. Dependence of clonal growth inhibition by 6-*O*-palmitoyl ascorbic acid (6P) on the action time: difference between duplicate administration and single shot of 6-*O*-palmitoyl ascorbic acid. 80 or 100 μM of 6-*O*-palmitoyl ascorbic acid were administered to A549 cells monodispersed on the dishes, but not to the control cells. At treatment time indicated, the spent medium was replaced by fresh medium with (+6P) or without (−6P) 6-*O*-palmitoyl ascorbic acid. The colonies were developed for 13 days. The ratio of colony number for each dish versus that for the control dish treated all the time with the medium containing no 6-*O*-palmitoyl ascorbic acid is expressed as a cloning efficiency (%). The bar represents S.D. for dishes in triplicate.

activated for 1–2 days with cell contact, because more cytotoxic effects were observed when the spent medium was replaced by fresh medium containing 6-*O*-palmitoyl ascorbic acid than by that containing no 6-*O*-palmitoyl ascorbic acid. Thereafter 6-*O*-palmitoyl ascorbic acid became inactive, because cytotoxic effects were temporally saturated for replacement by fresh medium containing no 6-*O*-palmitoyl ascorbic acid. The time-action curves for two doses of 6-*O*-palmitoyl ascorbic acid were similar in shape, although the inhibitory effect of 100 μM 6-*O*-palmitoyl ascorbic acid was more marked than that of 80 μM 6-*O*-palmitoyl ascorbic acid. This difference between two doses is reproducible, and is owing to the characteristics of dose-response curves of 6-*O*-acyl ascorbic acid that the cytotoxicity becomes marked abruptly when doses exceed the threshold concentration [13]. The action time curve of 6-*O*-palmitoyl ascorbic acid resembles that of ascorbic acid under the similar conditions [12]. The results indicate that potentiation of cytotoxic activity of 6-*O*-palmitoyl ascorbic acid is not caused by an increase in the stability or duration time of the cytotoxic action.

Antagonization of active oxygen species-scavenging enzymes against cytotoxic action of 6-O-palmitoyl ascorbic acid

Growth-inhibiting activity of 6-*O*-palmitoyl ascorbic acid to Ehrlich ascites tumor cells was slightly reduced by addition of native catalase or SOD alone but not by H_2O_2/3-amino-1,2,4-triazole-inactivated catalase, H_2O_2-inactivated SOD or the heat-denatured enzymes as shown by cell growth assay (Fig. 3). In contrast, the activity of ascorbic acid was completely lost by native catalase but not specifically inactivated catalase or SOD, or native SOD. Heat-denatured catalase or SOD rather slightly promoted the activity of ascorbic acid. This is probably due to transition metal ions contained in the enzymes, because ascorbic acid acquires an additional inhibitory activity in combination with metal ions [2,7,8]. Thus the activity of ascorbic acid is attributable exclusively to hydrogen peroxide initially produced, whereas hydrogen peroxide or superoxide alone is not responsible for the whole activity of 6-*O*-palmitoyl ascorbic acid.

The cytocidal activity of ascorbic acid was as low as inducing 64% metabolic death of human lung carcinoma A549 cells even at a concentration of 60 mM as shown by dye exclusion assay (Fig. 4). However, 2 mM 6-*O*-palmitoyl ascorbic acid induced the metabolic death as incident as 89%. At such high concentrations also, the similar results to those obtained by cell growth assay (Fig. 3) were shown: the activity of ascorbic acid was antagonized by catalase but not SOD, although 6-*O*-palmitoyl ascorbic acid was significantly inactivated by catalase or SOD alone. However, 6-*O*-palmitoyl ascorbic acid was markedly inactivated by combined addition of both catalase and SOD. These antagonistic effects of SOD and catalase on the cytocidal activity were not observed for the inactivated or heat-denatured enzymes. The results indicate that the cytocidal activity of 6-*O*-palmitoyl ascorbic acid is at least partly associated with both hydrogen peroxide and superoxide yielded at the early stage.

SOD, by binding metal ions, can often slow ascorbic acid oxidation [16]. Catalase plus SOD may inhibit the rate of autooxidation of 6-*O*-palmitoyl ascorbic acid in the medium used. The possibility was examined by iodine method (Table

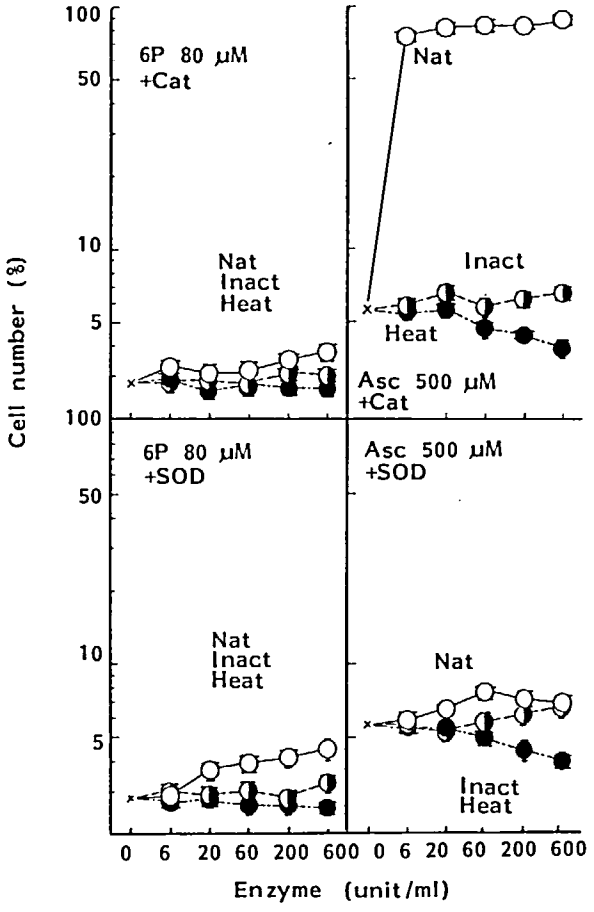

Fig. 3. Effects of scavenging enzymes on growth-inhibitory activity of ascorbic acid (Asc) or 6-*O*-palmitoyl ascorbic acid (6P). H_2O_2/3-amino-1,2,4-triazole-inactivated (Inact) catalase (Cat), H_2O_2-inactivated SOD, or the native (Nat) or heat-denatured (Heat) enzymes of indicated concentrations were administered to Ehrlich cells, which then received 500 μM ascorbic acid or 80 μM 6-*O*-palmitoyl ascorbic acid. After 5 days of treatment, cells were counted. The ratio of number of treated cells versus that of untreated cells is expressed as a cell number (%).

I). 6-*O*-palmitoyl ascorbic acid was more resistant against autooxidation than ascorbic acid, suggesting that the ene-diol group at C-2–C-3 positions of ascorbic acid is more difficultly oxidated in a micelle-like aggregate form of 6-*O*-palmitoyl ascorbic acid emulsified than in an isolated ascorbic acid molecule. Catalase plus SOD appreciably inhibited autooxidation of ascorbic acid but not that of 6-*O*-palmitoyl ascorbic acid under the similar conditions. The results confirm that the marked decrease in the cytotoxicity of 6-*O*-palmitoyl ascorbic acid by SOD plus catalase (Fig. 4) is mainly due to scavenging both superoxide and hydrogen peroxide generated at the early stage.

Counteraction of scavengers for singlet oxygen and hydroxyl radical against growth-inhibitory activity of 6-O-palmitoyl ascorbic acid

A diversity of scavengers were added to Ehrlich tumor cells or neuroblastoma NAs-1 cells, which

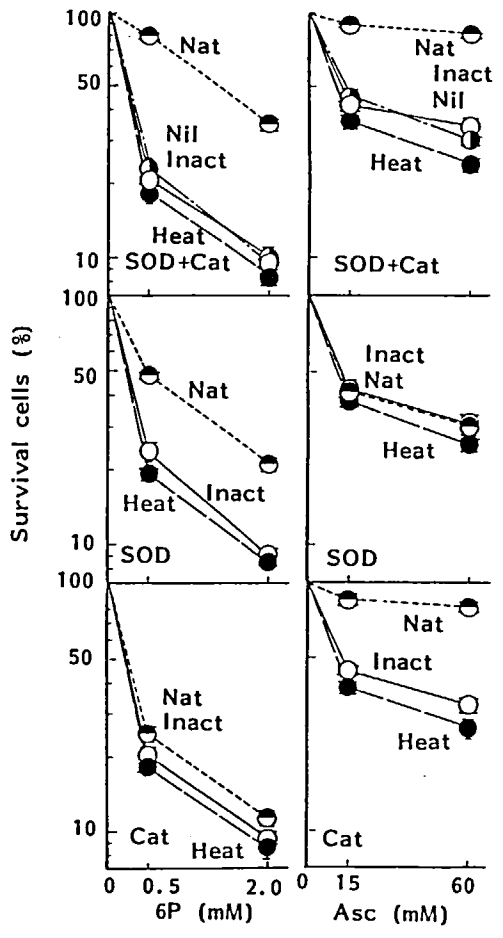

Fig. 4. Counteraction of scavenging enzymes against cytocidal activity of ascorbic acid (Asc) or 6-*O*-palmitoyl ascorbic acid (6P). Native (Nat) catalase (Cat), SOD (600 unit/ml), or their specifically inactivated (Inact) or heat-denatured (Heat) form dissolved in MEM/10% FCS was added to Ehrlich cells. For the control experiment, the medium without any enzymes (Nil) was added. The cells then received 6-*O*-palmitoyl ascorbic acid or ascorbic acid or indicated doses. After 48 h of treatment, the cytocidal activity was determined by dye exclusion assay. The bar represents S.D. of experimental values in triplicate.

then received 6-*O*-palmitoyl ascorbic acid. Thereafter alteration of the activity of 6-*O*-palmitoyl ascorbic acid was examined by cell growth assay (Fig. 5). The activity of 6-*O*-palmitoyl ascorbic acid was counteracted markedly by hydroquinone, slightly by 1,4-diazabicyclo-2,2,2-octane and significantly by sodium azide, respectively. All the three scavengers are usually used for quenching singlet oxygen. On the other hand no decrease in the activity of 6-*O*-palmitoyl ascorbic acid was observed for any hydroxyl radical scavengers such as D-mannitol, butylated hydroxytoluene, benzoate, ethanol, L-histidine and DL-α-tocopherol, and for another singlet oxygen scavenger bilirubin, which was not sufficiently soluble even with the aid of a trace of organic solvent. No growth inhibition was induced without 6-*O*-palmitoyl ascorbic acid at concentrations of the scavengers used, which were as high as effective for scavenging under the usual conditions [18]. Thus, the enhanced growth-inhibitory activity of 6-*O*-palmitoyl ascorbic acid may be associated with singlet oxygen but not with hydroxyl radical.

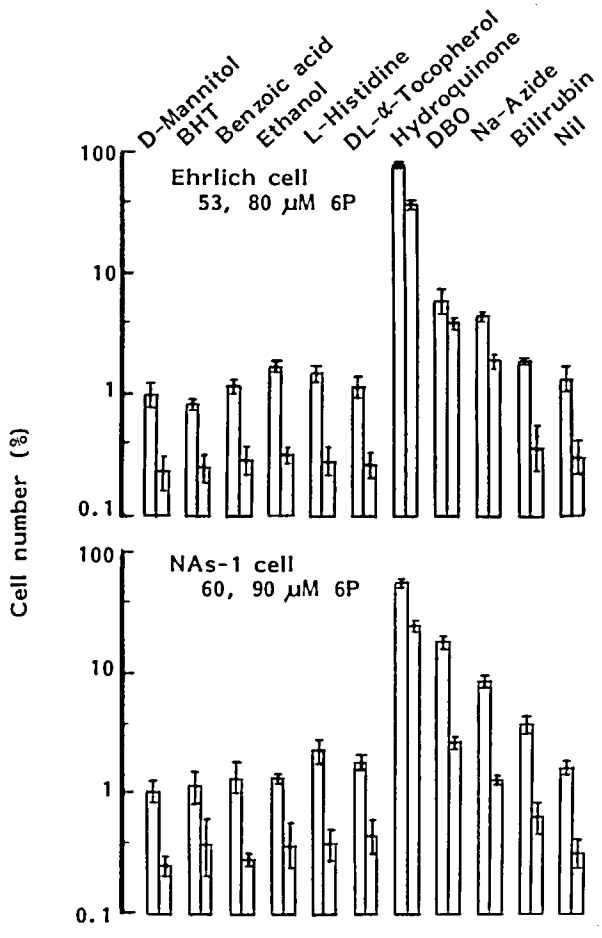

Fig. 5. Antagonizing effects of scavengers on growth-inhibitory activity of 6-*O*-palmitoyl ascorbic acid (6P). A variety of scavengers were added to Ehrlich cells or NAs-1 cells: 5 mM D-mannitol, 4 μM butylated hydroxytoluene (BHT), 0.4 mM benzoic acid, 0.5 mg/ml ethanol, 2 mM L-histidine, 90 μM DL-α-tocopherol, 1 μM hydroquinone, 15 μM 1,4-diazobicyclo-2,2,2-octane (DBO), 0.5 mM sodium azide and 8 μM bilirubin. For the control experiment, the cells received no scavengers (Nil). 6-*O*-palmitoyl ascorbic acid was then administered to the cells; 53 or 80 μM for Ehrlich cells and 60 or 90 μM for NAs-1 cells. The cell numbers for higher and lower doses of 6-*O*-palmitoyl ascorbic acid are indicated by the right and left columns, respectively. The activity of 6-*O*-palmitoyl ascorbic acid was determined by cell growth assay. The bar represents S.D. of experimental values in triplicate.

TABLE I

EFFECT OF CATALYSE PLUS SOD ON AUTOOXIDATION OF ASCORBIC ACID OR 6-*O*-PALMITOYL ASCORBIC ACID

3 ml of MEM/10% FCS containing 80 μM ascorbic acid or 6-*O*-palmitoyl ascorbic acid was retained at pH 7.2 and 37°C in a 60-mm dish. Catalase plus SOD (600 unit/ml) were previously added to some dishes. At indicated times, unoxidated compounds were quantified by iodine-starch method. Dishes in triplicate were determined for each time.

	Catalase + SOD	Unoxidated compd. (% ± S.D.)			
		Time (day)			
		0	2	4	7
Ascorbic acid	−	100±1	77±2	58±2	48±3
	+	99±2	85±1	70±2	51±4
6-*O*-Palmitoyl	−	100±0	95±0	93±1	91±1
	+	100±2	96±1	94±1	91±2

Discussion

The present study demonstrates that enhanced cytotoxic activity of 6-*O*-palmitoyl ascorbic acid over ascorbic acid is attributed to altered production of the active oxygen species but not to prolonged duration time of the cytotoxic action (Fig. 2). Both hydrogen peroxide and superoxide are yielded from 6-*O*-palmitoyl ascorbic acid, and they function synergistically to exhibit the cytotoxic activity as shown by reduction of 6-*O*-palmitoyl ascorbic acid activity by combination of catalase and SOD but not by either enzyme alone (Figs. 3

and 4). In contrast, hydrogen peroxide initially produced is exclusively responsible for the activity of ascorbic acid, which is irreversibly degraded to a variety of unstable reductones via diketogulonic acid [19]. Therefore, hydrogen peroxide may be derived from an ene-diol group of ascorbic acid moiety of a 6-*O*-palmitoyl ascorbic acid molecule. Alternatively superoxide may be derived from palmitoyl moiety of a 6-*O*-palmitoyl ascorbic acid molecule, whereas free palmitic acid or its methylester is less cytotoxic [13].

Singlet oxygen may be also produced from 6-*O*-palmitoyl ascorbic acid as suggested by marked diminution of 6-*O*-palmitoyl ascorbic acid activity by singlet oxygen scavenges (Fig. 5). Singlet oxygen in addition to hydroxyl radical is anticipated to be produced by Haber-Weiss reaction between hydrogen peroxide and superoxide [20,21]. Furthermore, cytotoxic activity of 6-*O*-palmitoyl ascorbic acid is more markedly inhibited by singlet oxygen scavengers than by a scavenger for hydrogen peroxide, superoxide or hydroxyl radical, aside from combined addition of two scavengers (Figs. 3–5). Therefore, the singlet oxygen from 6-*O*-palmitoyl ascorbic acid is suggested to be produced by transformation of both hydrogen peroxide and superoxide via cascade reacitons of disproportion [22]. Singlet oxygen is too short-lived to react with biocomponents distant from the production locus, although the lifespan is long in a hydrophobic microenvironment such as biomembranes [23]. Singlet oxygen can be scavenged by low molecular mass-compounds such as carotenoid and tocopherol bound to the biomembrane and ascorbic acid in the cytosol [22], but not specifically scavenged by the detoxicating enzymes of the living body. In contrast, hydrogen peroxide or superoxide is quenched primarily by glutathione peroxidase or SOD in vivo, respectively [18,24,25]. Moreover, superoxide from ascorbic acid or ascorbyl radical is also scavenged by SOD or ascorbyl radical reductase, respectively [26–28]. It is therefore suggested that the increased cytotoxicity following acylation of ascorbic acid is primarily attributed to conversion to some active oxygen species which are not specifically detoxicated by scavenging enzymes.

We have found that, by combination with hyperthermic treatment at 42°C, 6-*O*-palmitoyl ascorbic acid but not ascorbic acid enhances inhibitory activity to DNA synthesis in Ehrlich ascites tumor cells [29]. Furthermore, we have also found that ascorbic acid does not inhibit the activity of ornithine decarboxylase, which is a rate-limiting enzyme for polyamine biosynthesis and promptly increases prior to DNA synthesis [30–32], whereas 6-*O*-palmitoyl ascorbic acid markedly inhibits the enzyme activity post-transcriptionally (unpublished results). It remains to be analyzed how altered production of active oxygen species from 6-*O*-palmitoyl ascorbic acid is associated with polyamine biosynthesis or hyperthermia.

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