

Reduction of UVB/A-Generated Free Radicals by Sodium *L*-Ascorbyl-2-Phosphate in Cultured Mouse Skin

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The quenching abilities of sodium *L*-ascorbyl-2-phosphate (APS) and ascorbic acid 2-glucose (AG) against UVB/A-generated free radicals in cultured mouse skin were investigated using electron spin resonance (ESR). The relation between their quenching ability and protective effects against photodamage were also compared to those of ascorbic acid (AsA) pretreatment. Both APS and AG were able to scavenge UVB/A-generated hydroxyl radicals under aqueous conditions (pH 7.2) in a manner similar to that seen with AsA; however, APS was a more effective scavenger than AG. Similar results were obtained *ex vivo*. Both derivatives could protect skin from UVB/A-induced photodamage, as determined by a reduction in the presence of sunburn cells and DNA fragmentation. However, AsA pretreatment had the weakest protective effect, even though cutaneous, its level was the highest among the three agents tested before irradiation. These results indicated that the superior protective effect of APS is related to its direct free radical scavenging ability, rather than to its conversion to AsA.

Key words — sodium *L*-ascorbyl-2-phosphate, electron spin resonance, free radical scavenging, ascorbic acid, ascorbic acid 2-glucoside, UVB/A irradiation

INTRODUCTION

Depletion of stratospheric ozone makes the upper atmosphere less of a barrier to ultraviolet (UV) light. Skin is very susceptible to UV radiation, with overexposure leading to premature aging and carcinogenesis.^{1,2} UVB/A irradiation damages living cells by generating reactive oxygen species (ROS), especially singlet oxygen (¹O₂) and hydroxyl radicals (•OH).³

Biological defense mechanisms against ROS include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and low molecular weight compounds such as glutathione, ascorbic acid (AsA), and tocopherols.⁴ It has been reported that AsA, which is widely distributed in the body is a scavenger of ¹O₂ and •OH *in vitro*.^{5,6} Taken together, these data suggest that AsA may play a role in protecting the skin from UV-induced damage. Since AsA is unstable in aqueous solution, stable derivatives

with greater cellular uptake kinetics have been developed for therapeutic purposes. The derivatives of AsA, such as its 2-*O*-phosphate ester (APS)⁷⁻⁹ and 2-*O*-glucoside (AG)¹⁰ are readily taken up by cells and converted into AsA by phosphatase and glucosidase, respectively. APS has been shown to protect cells from DNA damage and death.¹¹ We earlier demonstrated that APS prevented UV-induced skin damage¹ to cultured mouse skin cells. The protection with AsA afforded these cells may have been due to its scavenging of •OH and/or lipid radicals that formed as a direct or indirect result of UV-exposure, by the dephosphorylated form of APS; however, direct evidence for this was not obtained.

The electron spin resonance (ESR) method has been widely used to directly and nondestructively measure ROS generated in biological tissues.¹² Previous ESR studies in skin examined the ROS scavenging properties of endogenous AsA,¹³⁻¹⁶ as well as other antioxidant compounds such as topically Desferal (an iron chelator) and tocopherol. *In vitro* experiments showed that ascorbyl palmitate, a fat soluble synthetic ester of AsA, was an efficient •OH radical scavenger.¹⁷

In the present study, we investigated the ability

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of the AsA derivatives, APS and AG, to quench free radicals *in vitro* and *ex vivo*.

MATERIALS AND METHODS

Reagents — APS and AG were obtained from Showa Denko K.K. (Tokyo, Japan), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Sodium *L*-ascorbic acid was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec (Tokyo, Japan). 4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (4H-TEMPO) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals — Female hairless mice (SKH-hr1; Sankyo Laboservice Inc., Tokyo, Japan), 5 weeks old were used in this study. They were housed and maintained under standard conditions (fluorescent light 12 hr per day, room temperature of 23°C and a relative humidity of 45–55%). The protocols for the animal experiments were approved by the Committee on Ethics of Animal Experiments at the Kyoritsu University of Pharmacy.

Skin Cultures — Skin cultures were set up according to the method described by Nakayama *et al.*⁹ Briefly, sterile lens paper was placed on 2 sheets of sterile nylon mesh with the tail end of the paper immersed in liquid medium (2 ml) in a plastic dish (3.5 cm). Squares of skin (2 × 2 cm), collected under sterile conditions from the backs of 5-week-old female hairless mice, were placed on top of the lens paper with the dermis facing the medium.

UVB/A Irradiation — The epidermal side of the cultured mouse skins was irradiated with UVB/A at a mean range of 20 kJ/m², using a UI-501C system (JEOL, Tokyo, Japan). For the UV radiation experiments, visible light and wavelengths below 280 nm were filtered out using a UV-D35 filter (JEOL). The UVB lamp used in this experiment emitted light at an efficiency of 63.3% in the 291–320 nm region and an efficiency of 36.7% in the 321–380 nm region. Exposure time was approximately 2 min (20 kJ/m²) and no heat was detectable during irradiation.

•OH Measurement *in Vitro* — Test solutions contained 92 mM DMPO and various concentrations of AsA or its derivatives in 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer, pH 7.2. These solutions were transferred into a quartz flat cell and positioned in the

cavity of an ESR detector for analysis. After UVB/A irradiation, ESR spectra were obtained at room temperature using a JES-RE1X (JEOL) at the following settings: microwave power, 4.0 mW; magnetic field, 335.0 ± 5 mT; sweep time, 2 min; modulation frequency, 100 kHz; time constant, 0.3 sec. Signal intensities were quantified by comparing the height of the first signal of the quartet of the DMPO-OH spin adducts in relation to the signal intensity of a Mn²⁺ standard. To determine whether AsA and its derivatives caused decay of the spin adducts (DMPO-OH), we conducted additional experiments using 4H-TEMPO, which is more stable than DMPO-OH. A solution containing 10 μM 4H-TEMPO and various concentrations of AsA or its derivatives in 100 mM HEPES buffer (pH 7.2) was transferred into a quartz flat cell and positioned in the ESR cavity for analysis. Signal intensities were quantified by comparing the height of the first signal of the triplet of the 4H-TEMPO spin adducts relative to the signal intensity of a Mn²⁺ standard.

Spin Trapping in Mouse Skins — Freshly obtained skin samples from hairless mice were incubated for 2 hr in media containing various concentrations of AsA or its derivatives in 100 mM HEPES buffer (pH 7.2) and were then washed twice with phosphate buffered saline (PBS). Samples pretreated with AsA or its derivatives were cut into 5 × 5 mm pieces for ESR analysis. Epidermal samples were incubated with DMPO (5 μl of 9.2 M DMPO) for 5 min at room temperature, after which they were blotted and placed in a tissue cell and positioned in the ESR cavity. After UVB/A irradiation, ESR spectra were obtained at room temperature.

HPLC Analysis — The concentrations of endogenous AsA and its derivatives were determined by HPLC using a reverse-phase column (for AsA; Shodex Asahipak NH2P-50 4E, 4.6 × 250 mm, for AsA-derivatives; Shodex NHpak J-411, 4.6 × 150 mm). The mobile phase consisted of 80% acetonitrile containing 0.1% H₃PO₄ for AsA, or 0.1 M KH₂PO₄ and 50% acetonitrile for the AsA-derivatives. The separation was performed at a flow rate of 0.8 ml/min at 40°C. AsA and its derivatives were analyzed by UV detection at 265 and 257 nm, respectively. The samples were homogenized in a solution of 0.1 M HEPES buffer pH 7.2 (1 g tissue wet weight/20 ml) using a Polytron homogenizer (Kinematica; CH-1600, Zurich, Switzerland). Homogenates were added to 0.1% 1,4-dithioerythritol and 2 volumes of acetonitrile containing 0.15% H₃PO₄, and were then centrifuged.

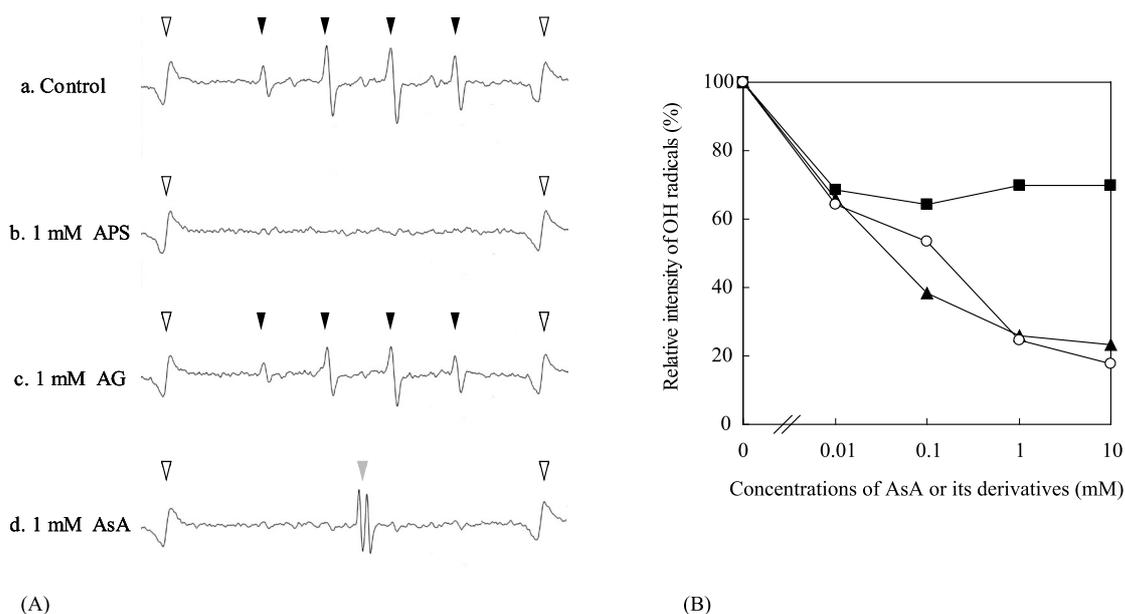


Fig. 1. UV-Generated \bullet OH Scavenging by AsA and its Derivatives

A) Signals of the DMPO-OH spin adducts (▼), ascorbic acid radicals (▽), and Mn^{2+} (▽) standard. B) Intensity of hydroxyl radicals scavenging ability of AsA, APS and AG. The data represents the mean of 2 experiments. ▲, APS in HEPES buffer, pH7.2. ■, AG in HEPES buffer, pH7.2. ○, AsA in HEPES buffer, pH7.2.

Twenty μ l of the supernatant was injected onto the column.

Histochemical Analysis — Skin samples were incubated for 2 hr in media containing various concentrations of AsA or its derivatives. The samples were then washed twice with PBS and the media were replaced with PBS, after which UVB/A irradiation (20 kJ/m²) was performed. Following irradiation, the skin samples were cultured for 22 hr in Dulbecco's modified Eagle medium (phenol red free) without AsA or its derivatives. Cultured skin samples were then fixed with 10% formaldehyde in 0.1 M sodium phosphate buffer for 15 min. For microscopy, specimens were dehydrated in ethanol, embedded in paraffin, and then stained with 1% eosin and hematoxylin and eosin (HE). Photographs of these sections were examined and counts made of the number of sunburn cells — distinguishable by their dense, dark-staining (darker than neighboring keratinocytes), and irregular nuclei — in 0.2 mm of epidermis at three sites within each skin sample.

To detect DNA degradation, sections of skin were deparaffinized in xylene and ethanol, and were then rehydrated and incubated with proteinase K (Sigma Chemical Co.) to strip away nuclear proteins. Degraded DNA was detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method,¹⁸⁾ which was modified so that terminal transferase directly

incorporated fluorochrome-labeled dUTP (TaKaRa Biomedicals, Tokyo, Japan). The TUNEL-stained skin samples were visualized with a fluorescence microscope (Leica MPS60, Leica AG, Heerbrugg, Switzerland) and photomicrographs were taken. The number of sunburn cells was expressed as a percentage of the total number of keratinocytes in 0.2 mm of epidermis.

Statistics — Data are expressed as the mean \pm S.D. Statistical analyses were performed using a one-way analysis of variance (ANOVA), followed by Bonferroni test. Differences of $p < 0.05$ were considered to be statistically significant.

RESULTS

UVB/A-Generated \bullet OH Scavenging by AsA and its Derivatives *in Vitro*

When HEPES buffer (pH 7.2) was exposed to UVB/A irradiation, \bullet OH signals immediately appeared (Fig. 1A-a), and these signals disappeared when AsA or APS was added at a concentration of 1 mM (Fig. 1). These signals were definitively identified as DMPO-OH adducts based on their g -value (data not shown). The relative signal intensity was expressed as a percentage of the irradiated control. UVB/A-generated \bullet OH radicals were scavenged in a dose-dependent manner by APS (Fig. 1B). At a

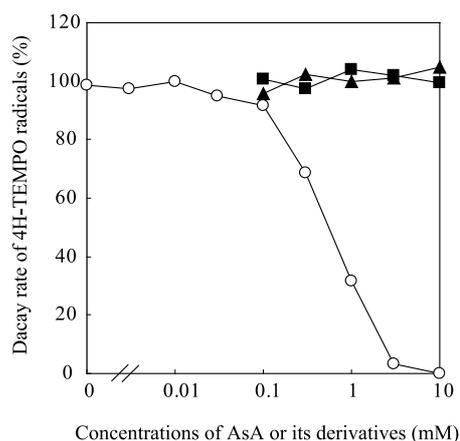


Fig. 2. Influence of APS, AG and AsA on 4H-TEMPO Spin Adducts

▲, APS; ■, AG; ○, AsA.

concentration of 0.1 mM, quenching level by APS was similar to that of AsA. Very little AsA released from APS in this experiment (data not shown). The results of these *in vitro* experiments suggested that APS could quench $\cdot\text{OH}$ directly. It was estimated that APS (10 mM) reacted with the $\cdot\text{OH}$ at least one order of magnitude faster than with DMPO (92 mM).

Influence of APS, AG and AsA on 4H-TEMPO Spin Adducts

It is well known that AsA decays spin adducts. Thus, to determine whether APS and AG can also cause the decay of spin adducts, 4H-TEMPO was used instead of DMPO-OH. The signal intensity of 4H-TEMPO was expressed as the peak height of the first signal of the triplet of the 4H-TEMPO spin adducts relative to the signal of the Mn^{2+} standard (data not shown). As shown in Fig. 2, the intensity of the 4H-TEMPO spin adducts signal was unchanged in the presence of either APS or AG at concentrations of 0.1–10 mM. These results indicated that neither of these derivatives had any effect on spin adduct formation. Thus, the high $\cdot\text{OH}$ quenching ability seen with 1 and 10 mM AsA was probably an overestimation (Fig. 1).

Incorporation of AsA Derivatives in Cultured Skin and their Conversion to AsA

The concentration of AsA and its derivatives in cultured skin was determined before irradiation. After 2 hr of treatment, both derivatives, APS and AG were incorporated into the skin in a concentration-dependent manner and reached approximately 25% of the administered concentration (Fig. 3). Ap-

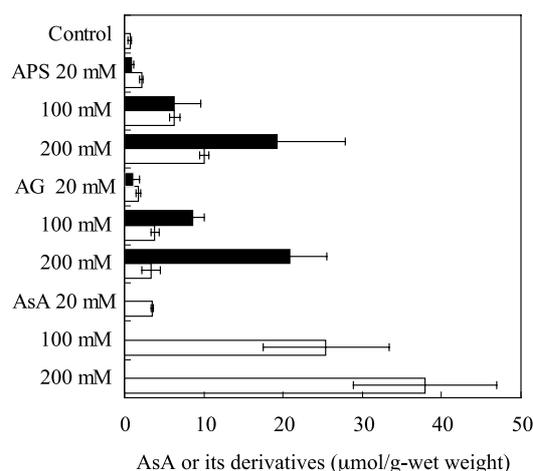


Fig. 3. Concentration of AsA and its Derivatives in Cultured Skin Samples Treated with 20–200 mM AsA, APS or AG

The concentrations of AsA (open bars) and its derivatives (closed bars) were analyzed by HPLC using a reverse-phase column. Each bar represents the mean \pm S.D. of 6 samples from 3 mice.

proximately 5% of the administered amounts of APS and AG were converted to AsA under the skin. APS and AG were not converted to AsA in this experimental condition (data not shown). When skin samples were pretreated with 100 mM AsA, APS or AG, their AsA concentration increased by 35-, 6- and 3.5-fold, respectively, relative to untreated skin.

Radical Scavenging Effects of APS, AG and AsA in Cultured Mouse Skin

Following UVB/A irradiation, DMPO spin adducts of hydroxyl and ascorbate radicals were clearly observed in mouse skin samples (Fig. 4A). As shown in Fig. 4B, the DMPO spin adducts generated by UVB/A irradiation in mouse skin were scavenged by pretreatment with AsA and its derivatives. However, before irradiation, pretreated skin accumulated a sufficient AsA concentration to cause the decay of the DMPO spin adduct. Therefore, it appears that the initial estimates of free radical scavenging activity may have been overestimated. In our *in vitro* experiments, 10 mM AsA resulted in complete decay of the spin adducts (Fig. 2).

Protective Effects of APS and AG Against UVB/A-Induced Skin Damage

Destruction of the stratum corneum and the formation of epidermal cells with dwarfed nuclei were observed in sections of HE-stained control skin that had been cultured for 24 hr. Cultured skin irradiated with UVB at 20 kJ/m² displayed many pyknotic and

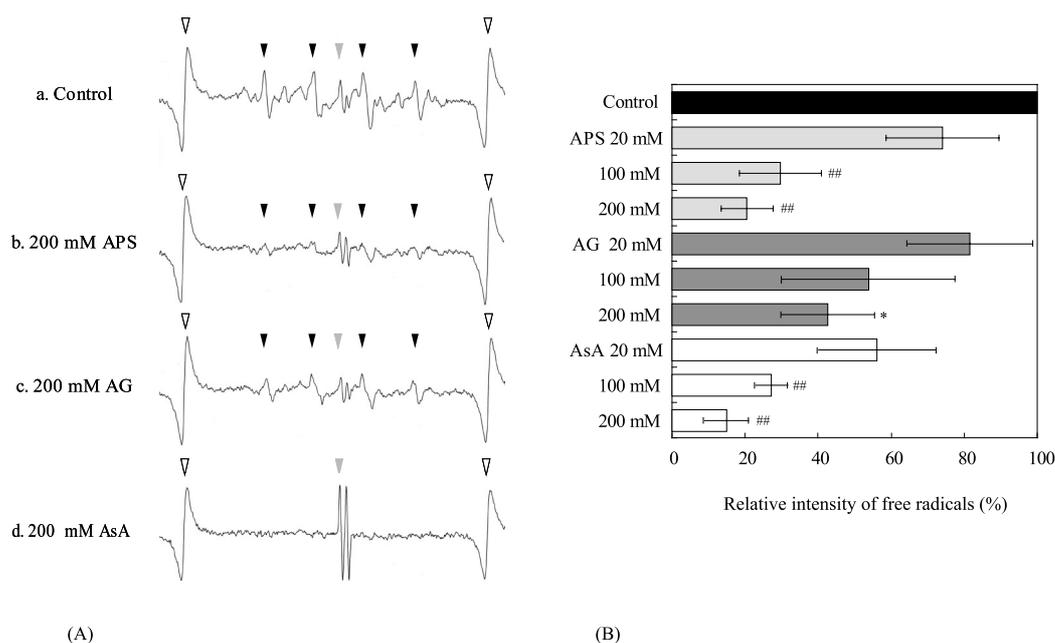


Fig. 4. Free Radical Scavenging Abilities of APS, AG and AsA in Mouse Skin

A) Signals of DMPO-OH spin adducts (▼), ascorbic acid radicals (▽) and Mn^{2+} (▽) standard are shown. B) Effects of pretreatment with AsA, APS, and AG on $\bullet OH$ scavenging in mouse skin. Each bar represents the mean \pm S.D. of 6 samples. # $p < 0.05$ and ## $p < 0.01$. ■, Irradiated skin (control). □, Pretreated with APS, irradiated. ■, Pretreated with AG, irradiated. □, Pretreated with AsA, irradiated.

denucleated epidermal cells indicative of sunburn cells (Fig. 5A). These cells were reduced in number in irradiated cultured skin that was pretreated with APS or AG for 2 hr, though no such reduction was seen in the AsA pretreated skin.

Examination of DNA degradation using the TUNEL assay (Fig. 5B) revealed several lightly fluorescence-labeled nuclei in the epidermal cells of control, cultured skin. In the UVB-irradiated skin TUNEL-positive fluorescent nuclei were clearly observed, suggesting that such treatment had caused DNA strand breaks. In the cultured skin pretreated with APS for 2 hr, the number of epidermal cells with fluorescence-labeled nuclei was reduced, suggesting that this compound inhibited the endogenous degradation of DNA. In the AsA pretreated skin, however, the number of TUNEL-positive cells approximated that seen in the irradiation control.

To quantify the above results, the number of sunburn cells in the above-mentioned photomicrographs was expressed as a percentage of the total number of epidermal cells in 0.2 mm of epidermis (Fig. 6). In control skin incubated for 24 hr, sunburn cell formation was observed in approximately 10% of cells. This number increased after UVB/A irradiation reaching approximately 60% in skin that was UVB/A irradiated at 20 kJ/m². In both skin pretreated with

APS and with AG for 2 hr, this increase in sunburn cell formation was inhibited significantly. At 20 kJ/m², there was no significant difference in the inhibition ratio between AsA treated and untreated skin.

The protective effect of APS was greater than that of AG. However, AsA pretreatment had less of a protective effect than the other two test compounds even though the cutaneous AsA concentration in AsA-pretreated skin was higher.

DISCUSSION

Our results showed that APS and AG have a direct, free radical scavenging effect and could prevent UVB/A-induced skin damage. We previously reported that APS pretreatment prevented against UVB-induced skin damage^{9,19}) and hypothesized that this protective effect of APS might be due to the scavenging of ROS by AsA that was converted from APS, since the AsA concentration in the APS pretreated skin increased in a time-dependent manner. However, the results of our current study suggested that the protective effect of APS may be due to its direct scavenging ability rather than to its conversion to AsA.

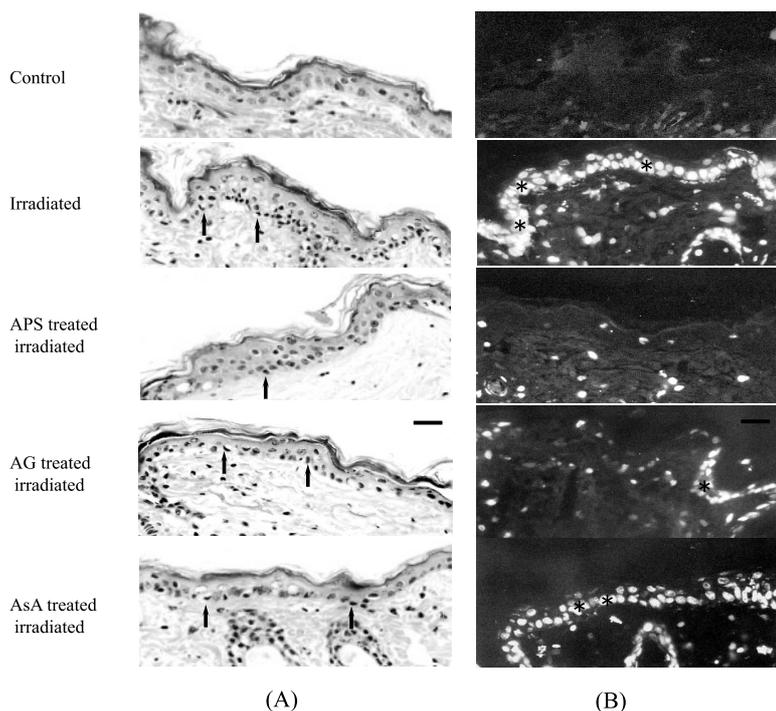


Fig. 5. Photomicrographs of Cultured Mouse Skin Stained with 1% HE (A), and by the TUNEL Method (B), 24 hr after UVB/A Irradiation at 20 kJ/m²

→: sunburn cells, *: TUNEL positive nuclei.

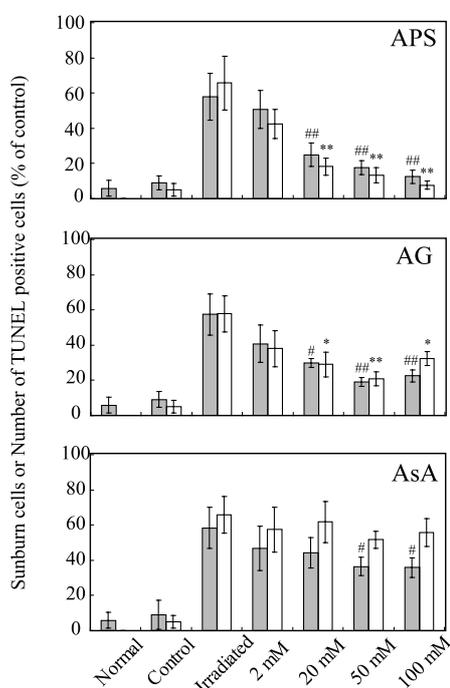


Fig. 6. Protective Effects of APS, AG and AsA on Sunburn Cell Formation and DNA Fragmentation Induced by UV Irradiation

The number of sunburn (open bars) and TUNEL-labeled cells (closed gray bars) in 0.2 mm of epidermis from cultured mouse skin are indicated. Each bar represents the mean \pm S.D. of 6 pieces of skin from each of 3 mice. #*p* < 0.05 and ##*p* < 0.01 relative to untreated, irradiated mouse skin.

In general, the ene-di-ol groups at both C-2 and C-3 (particularly C-2 which has a high electron density) mediate the antioxidant activity of AsA. Because the phosphoric group is located on 2-OH residue within APS, the OH residue at C-3 could exchange electrons with free radicals.¹⁸⁾ However, the precise mechanism underlying the free radical scavenging activity of APS has not yet been conclusively identified. In an ESR spin trapping study,¹⁷⁾ Perricone *et al.* demonstrated that *L*-ascorbyl-6-palmitate (0.1–5 mM), a fat-soluble synthetic ester of AsA, could scavenge \bullet OH formed *in vitro* by the Fenton reaction or by γ -irradiation.

It is well known that ascorbate can cause the decay of DMPO-OH spin adducts. In our experiments using 4H-TEMPO, neither of the AsA derivatives affected the decay of DMPO spin adducts, even at concentrations as high as 10 mM. However, at 0.1, 1 and 10 mM, AsA decreased spin adduct levels by 8, 70 and 100%, respectively. Therefore, it is likely that the \bullet OH scavenging effects of AsA at concentrations of 1 and 10 mM were overestimated. The addition of 1 mM AsA resulted in a 70% decay of spin adducts relative to skin that was exposed to UVB/A irradiation alone (Fig. 2). However, in cultured skin that had accumulated AsA to a concentra-

tion of 25 mM AsA (following pretreatment with 100 mM AsA) the quenching effect was limited to 30% of that seen in control skin exposed to irradiation only. In APS and AG pretreated skin, the AsA levels that resulted from the conversion from derivatives was less than 10 mM. Most of the reductions in the levels of DMPO spin adducts did not seem to result from decay; thus, it appears that APS and AG do, in fact, scavenge UVB/A-generated free radicals in mouse skin.

Pretreatment with APS and AG significantly protected skin samples from photodamage, as indicated by their reduced numbers of sunburn cells and DNA fragmentation. In previous studies, we similarly observed that UVB-induced lipid peroxidation in cultured skin was inhibited by APS-pretreatment.^{9,19)} However, even though pretreatment with AsA resulted in the highest cutaneous AsA concentrations of the three AsA-related compounds tested, it provided the least effective protection against skin photodamage. Therefore, the protective effects of APS and AG were likely due to their direct quenching of free radicals rather than to their conversion to AsA.

It was reported that the AsA levels in the epidermis *in vivo* were reduced by UV irradiation²⁰⁾ and that the administration of AsA immediately before UVB irradiation inhibited radiation-induced onset of erythema, sunburn cell formation²¹⁾ and the formation of 8-hydroxydeoxyguanosine.²²⁾ In our study, even though AsA pretreated skin contained the highest AsA levels before irradiation, AsA exerted the weakest protective effects of the three AsA compounds tested. Alternatively, this might have been due to increased AsA radical formation following UVB/A-irradiation, which is dependent on cutaneous AsA concentrations. Buettner and Jurkiewicz²³⁾ observed that UV irradiation (330 nm) increased endogenous ascorbate free radical concentrations in hairless mice. The AsA radical has a relatively long half-life compared to other free radicals, such as hydroxyl, peroxy, alkoxy and carbon-centered lipid free radicals.²³⁾ Moreover, cutaneous AsA is oxidized to its free radical form by UVB-irradiation, which can be monitored by ESR.¹⁹⁾ From these results it appears that excess levels of AsA radicals could accelerate cutaneous photodamage. Indeed, *in vivo* experiments demonstrated that 2% AsA enhanced the induction of carcinogenesis induced by butylated hydroxyanisole in rodents.²⁴⁾ Although we cannot readily explain this discrepancy, it appears that APS is better suited than AsA for protecting skin against

daily UV-exposure.

In conclusion, our *in vitro* and *ex vivo* experiments showed that APS itself acts as a scavenger of UVB/A-generated free radicals in mouse skin. Furthermore, APS- and AG-pretreatment protected skin from UVB/A-induced damage as determined by the number of sunburn cells and degree of DNA fragmentation. These results suggest that the superior protective effect of APS is due to its direct free radical scavenging effects, rather than to its conversion to AsA. The administration of high concentrations of AsA did not protect mouse skin from photodamage.

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