Protective Effects of Sodium-L-ascorbyl-2 Phosphate on the Development of UVB-Induced Damage in Cultured Mouse Skin

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The protective effect of sodium-L-ascorbyl-2 phosphate (As-2P), a stable form of ascorbic acid (AsA), against photodamage induced by a single dose of UVB exposure (290—320 nm, Max 312 nm) was investigated using cultured mouse skin.

When the cultured skin was treated with various As-2P concentrations, the cutaneous AsA level increased in proportion to the As-2P concentration. After 3 h of incubation, the AsA level in the cultured skin treated with 2, 20 and 100 mM As-2P increased 1.03-, 2.17- and 6.27-fold, respectively, compared with that of the control skin. These results suggest that As-2P was transported into the cultured mouse skin where it was converted to AsA. After 3 h, the cutaneous AsA level in irradiated (20 kJ/m\textsuperscript{2}) skin was depleted to a half of that in the control skin. However, the level in skin pretreated with 20 mM As-2P was maintained within normal limits, even after 24 h. Pretreatment with 20 mM As-2P significantly prevented such photodamage as sunburn cell formation, DNA fragmentation and lipid peroxidation, which were caused by a single dose of UVB irradiation. These results suggest that the protective effect of 20 mM As-2P on UVB-induced cutaneous damage is due to the maintenance of a normal As level by conversion of As-2P to As in skin tissue.

Key words  sodium-L-ascorbyl-2 phosphate; UVB-irradiation; cultured skin; DNA fragmentation; lipid peroxidation; sunburn cell formation

A correlation has been observed between the reduction in atmospheric ozone concentration and increased levels of ultraviolet B (UVB) irradiation.\textsuperscript{1} UVB irradiation is suspected to exert significant adverse effects on health, including suppression of the immune system, cataract formation and melanoma and non-melanoma skin cancer. One of the causes of cutaneous damage induced by UVB irradiation is the generation of reactive oxygen species (ROS) in living cells.\textsuperscript{2,3} It is well known that ROS, such as superoxide radicals (O\textsubscript{2}-), hydroxyl radicals (·OH), singlet oxygen (O\textsubscript{2}·) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), are associated with skin cancers, cutaneous photoaging and many cutaneous inflammatory disorders.\textsuperscript{3}

Biological defense mechanisms against ROS-induced oxidative stress include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and low molecular weight compounds such as glutathione, ascorbic acid (AsA), and \textalpha;-tocopherol. Photoaging and skin diseases, such as UV-induced skin cancer and immunosuppression, are believed to be precipitated by exposure that exceeds the threshold levels of the defense functions. Among the antioxidants, it is known from \textit{in vitro} experiments that AsA, which is widely distributed in the body, is a scavenger of singlet oxygen and ·OH.\textsuperscript{4,5} Further, AsA acts to indirectly inhibit lipid peroxidation by reducing tocopherol radicals produced by the oxidation of \textalpha;-tocopherol.\textsuperscript{6} In fact, it has been reported that AsA levels in epidermal skin \textit{in vivo} are reduced by UV irradiation,\textsuperscript{7} and that the administration of AsA immediately before UVB irradiation inhibits the radiation-induced onset of erythema, sunburn cells\textsuperscript{8} and the formation of 8-hydroxydeoxyguanosine.\textsuperscript{9} These results suggest that in each case, AsA has a protective effect against UV radiation-induced skin damage, and that administration of AsA could alleviate damage caused by daily UV irradiation of the skin. However, AsA is normally unstable in aqueous solution, and because its cellular uptake is limited, stable derivatives that are better transported into cells have been developed. A derivative of AsA, such as its 2-\textalpha;-phosphate ester, is continuously taken up by cells and converted into AsA.\textsuperscript{10,11} Sodium-L-ascorbyl-2 phosphate (As-2P) is rapidly taken up into cells with an excellent accumulation profile.\textsuperscript{12} In earlier \textit{in vivo} experiments, we reported that intraperitoneally administered As-2P significantly inhibited UVB irradiation-induced lipid peroxidation and inflammation in mouse skin and retarded the formation of skin squamous cell carcinoma due to continuous irradiation.\textsuperscript{13}

In the present experiments, we investigated whether protective effects against UVB irradiation-induced damage, such as sunburn cell formation, DNA fragmentation and lipid peroxidation, could be conferred by the percutaneous permeation of As-2P, using cultured mouse skin.

MATERIALS AND METHODS

Materials  As-2P was supplied from Showa Denko Co. (Tokyo), and l-ascorbic acid sodium salt (AsA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals  Female hairless mice (SKH-hrl; Sankyo Laboratory Inc., Tokyo, Japan), aged 5 weeks at the beginning of the experiment, were used. They were housed under standard conditions (fluorescent light 12 h/d (08:00—20:00 h), room temperature 23 °C and relative humidity 45—55%) and fed a commercial diet and water \textit{ad libitum}. The protocol of the animal experiments was approved by the Committee of the Ethics of Animal Experiments of Kyoritsu College of Pharmacy.

Culture  In the present experiments, we used cultured hairless mouse skin instead of \textit{in vivo} studies for the following reasons: quantitative topical application in percutaneous per-
mention experiments using animals is difficult because the animals tend to lick off the test compound; sealing the test compound to the skin under tape or other means causes stress; and UVB irradiation-induced lipid peroxidation is difficult to detect. The culture method was as follows: lens paper was placed on 2 sheets of nylon mesh and the tail end of the paper was immersed in liquid medium in a plastic dish (Fig. 1). 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 epidermis facing the medium. A layer of Parafilm (P) was placed on top in order to prevent the preparation from drying out. O: dermis.

![Image](image.png)

**Fig. 1. Method of Organ Culture**

Lens paper (L) was placed on 2 sheets of nylon mesh (N) and the tail end of the paper was immersed in liquid medium (M) in a plastic dish. Squares of skin (2×2 cm) collected from hairless mice were placed on top of the lens paper with the epidermis (E) facing the medium. A layer of Parafilm (P) was placed on top in order to prevent the preparation from drying out. O: dermis.

content was measured by the method of Bradford, with bovine plasma globulin as the standard.

**Histochemical Observations of Sunburn Cell Formation and DNA Fragmentation** The cultured skins were fixed in 2.5% glutaraldehyde with 0.1 M sodium phosphate buffer for 15 min. For microscopy, the specimen was dehydrated with ethanol and embedded in paraffin. The sectioned specimen was then stained with 1% eosin and hematoxylin. Photographs were examined and counts made of the number of sunburn cells, which could be identified by a dense, irregular nucleus, darker than the nuclei of neighboring keratinocytes in 0.2 mm of epidermis at three sites of the cultured skin. Microtomed sections of skin were deparaffinized by xylene and ethanol, rehydrated, and incubated with proteinase K (Sigma, St. Louis, MO, U.S.A.) to strip away any nuclear proteins. To detect DNA strand breaks, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method was modified so that terminal transferase directly incorporated fluorochrome-labeled dUTP (TaKaRa Biomedicals, Tokyo). The TUNEL-stained skin samples were visualized with a fluorescence microscope (Leica MPS60, Leica AG, Heerbrugg, Switzerland), then photomicrographs were taken. Photographs were made of the number of TUNEL-labeled cells in 0.2 mm of epidermis at three sites of the cultured skin. The numbers of sunburn cells and TUNEL-labeled cells were expressed as a percentage of total keratinocytes in 0.2 mm epidermis.

**Statistics** Data are expressed as the mean±S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the Bonferroni t-test. Differences of p<0.05 were considered to be statistically significant.

**RESULTS**

**Conversion of As-2P to AsA and Persistence in Skin Tissue** In skin culturing, the level of cutaneous AsA had gradually fallen in a time-dependent fashion. After 3, 6 and 24 h, the level had declined to approximately 85%, 78% and 50%, respectively (Fig. 2). Hence, taking into account this decrease in endogenous AsA levels, we irradiated cultured skin with UVB radiation within 3 h of culture. When the cultured skins were treated with various As-2P concentrations, the cutaneous AsA level increased in proportion to the As-2P concentration. After 3 h of incubation, the AsA levels in skins treated with 2, 20 and 100 μM As-2P increased to 1.03-, 2.17- and 6.27-fold, respectively, that non-treated skin. These results suggest that As-2P is converted to AsA in skin after percutaneous permeation and the normal AsA level is maintained for at least 24 h. When cultured skins were subjected to UVB irradiation at 20 kJ/m², after 3 h the levels of cutaneous AsA were depleted to half that in normal skins. Pretreatment with 2 μM As-2P had no protective effect against the irradiation-induced cutaneous AsA decrease. However, pretreatment with 20 and 100 μM As-2P prevented the decrease. Especially, the level in the skin pretreated with 20 μM As-2P was maintained within normal limits even after 24 h.

**Inhibitory Effects on Lipid Peroxidation** After 21 h, TBARS levels for cultured skin irradiated with UVB at 20 kJ/m² increased to double that of control skin (Fig. 3). The irradiation-induced increase in TBARS levels was significantly inhibited in cultured skin pretreated with 20 and
100 mM As-2P to a value that was somewhat lower than that observed for the control skin. This protective effect may be attributed to pretreatment with As-2P and subsequent conversion to AsA.

**Protective Effects against Sunburn Cell Formation and DNA Fragmentation** The number of sunburn cells with condensed or absent nuclei, as shown by the arrows in the photomicrograph, 24 h after the skin cultures were irradiated with UVB at 20 kJ/m² increased (Fig. 4a) compared to the control cells. For cells pretreated with 20 mM As-2P for 3 h, however, a decline in the number of sunburn cells was observed. A few TUNEL-positive cells were found in control epidermis (Fig. 4b). Labeled cells in the dermis appeared to be leukocytes with nonspecific stained cytoplasmic granules. TUNEL-labeled keratinocytes were observed in the UVB-irradiated epidermis. For cells pretreated with 20 mM As-2P for 3 h, the number of TUNEL-stained cells in UVB-irradiated skin decreased.

![Fig. 2. Time Course of the Conversion of As-2P to AsA in Cultured Mouse Skin](image)

- □, control cultured skin; ■, irradiated (20 kJ/m²); △, pretreated with 2 mM As-2P;
- ●, pretreated with 2 mM As-2P then irradiated; ○, pretreated with 20 mM As-2P;
- ●, pretreated with 20 mM As-2P then irradiated; ○, pretreated with 100 mM As-2P;
- ●, pretreated with 100 mM As-2P then irradiated.

![Fig. 3. Amount of Cutaneous TBARS Concentration in the Cultured Mouse Skin Pretreated with As-2P 3 h before UVB Irradiation](image)

\(^*\) p < 0.01 compared with non-cultured, normal skin; \(^*\) p < 0.05 relative to normal skin; \(^*\) p < 0.01 compared with irradiated cultured skin. Each bar represents the mean ± S.D. of 10 samples.

![Fig. 4. Photomicrographs of Cultured Mouse Skin Stained with 1% Hematoxylin and Eosin (a), or the TUNEL Method (b)](image)

N, normal skin; C, control cultured skin; UVB, irradiated (20 kJ/m²); As-2P UVB, pretreated with 20 mM As-2P then irradiated (20 kJ/m²). Bar = 50 μm.
The number of sunburn or TUNEL-stained cells in a 0.2 mm section of epidermis were counted on the photomicrographs. As shown in Fig. 5, the number of sunburn cells was 4-fold higher than that of controls, while the number of TUNEL-stained cells was 20-fold higher. For epidermal skin treated with 20 mW As-2P, the irradiation-induced increases were significantly inhibited with numbers closer to those found in the control samples. These results demonstrate that AsA, derived from the conversion of percutaneously-permeated As-2P, protects epidermal cells from sunburn cell formation elicited by irradiation.

DISCUSSION

When mouse skin was cultured in a medium containing As-2P, AsA concentration in the skin tissue increased and remained elevated for up to 24 h. Fujiwara et al. demonstrated that the amount of intracellular AsA was 7.2- to 9.0-fold larger in cells treated with As-2P. However, in the cells, no intact As-2P was detected. This may be a result of the continuous incorporation of As-2P through the skin, where it is converted to AsA as a result of dephosphorylation by acid phosphatase in the epidermis, then accumulated as AsA. In an earlier in vitro experiment using mouse skin epidermal tissue, we demonstrated that As-2P is transported into the epidermis in a concentration-dependent manner, where it is converted into AsA, then permeates outside the epidermis. Kanatake et al. also showed that As-2P is also taken up into cells in normal human epidermal keratinocytes (NHEK), where it is converted into AsA and accumulates; moreover, the accumulated AsA content level did not change over the ensuing 24 h period.

In situ experiments Kameyama et al. demonstrated that magnesium t-ascorbyl-2-phosphate (VC-PGM) cream was absorbed into human cadaver skin; the amount of radiolabeled VC-PGM in the epidermis plus dermis was 1.58%. In this study, after 3 and 6 h of incubation, approximately 1.5% and 4%, respectively, of the added As-2P was transported then converted to As in the cultured mouse skin. Differences in the methods of percutaneous permeation or differences in species may account for these different permeation ratios.

Dart et al. reported that administering AsA immediately before UVB irradiation inhibits the onset of irradiation-induced erythema and sunburn cells. Similarly, in the present experiment the number of sunburn cells due to UVB irradiation decreased after pretreatment with As-2P. Moreover, the number of TUNEL-stained cells by UVB irradiation decreased with As-2P pretreatment. While the question remains whether these sunburn cells correspond to TUNEL-stained cells, the results of the present experiment suggest that As-2P converted into As exerts a protective effect against irradiation-induced apoptosis. The finding that several cells with condensed nuclei were also observed in the control cultured skin may have been due in part to the culturing-induced reduction in the concentration of AsA (Fig. 5).

In vitro experiments, we have demonstrated that UVB-induced hydroxyl radical generation in mouse skin homogenates is scavenged by AsA addition, which is detected by electron spin resonance. Therefore, it is presumed that As-2P has a protective effect when it is converted in the serum or keratinocytes to AsA.

In the present experiment, the TBARS level in normal or irradiated cultured mouse skin coincided with data in our previous in vivo experiment. Thus, our organ culture method may be an available model for in vitro experiments.

In conclusion, we demonstrated that pretreatment with As-2P protects skins from UVB irradiation-induced skin damage, such as lipid peroxidation, sunburn cell formation and DNA fragmentation. We found, in an in vitro experiment using mouse cultured skin, that As-2P is transported into the cutaneous tissue where it was converted to AsA. This protective effect of As-2P may be due to the scavenging of reactive oxygen species by AsA that is produced by hydrolysis in the skin.

REFERENCES

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