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Preventive effects of phosphorylated ascorbate on ultraviolet-B induced apoptotic cell death and DNA strand cleavage through enrichment of intracellular vitamin C in skin epidermal keratinocytes

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Abstract

Mortality of mouse keratinocytes Pam212 that were irradiated with ultraviolet-B (UVB) was shown to be repressed by pre-irradiated administration with L-ascorbic acid (Asc) or more markedly with Asc-2-O-phosphate (Asc2P), but not with dehydroascorbic acid (DehAsc) or Asc-2-O-alpha-glucoside (Asc2G), although not repressed by post-irradiated administration. The cytoprotection by Asc2P was restricted against UVB below 5–20 mJ/cm^2, and exhibited markedly by administration for a period over 2 h, which may be caused by intracellular Asc that was accumulated via dephosphorylation of Asc2P and was increased, 6–24 h after, to levels above twice as abundant as those of Asc-administration. Pre-irradiated Asc2P-administration slightly repressed a DNA ladder-like electrophoretic pattern for UVB-irradiated keratinocytes, containing the histone-bound DNA fragments as shown by ELISA assay, and appreciably repressed the DNA-3′OH cleavage terminals as shown by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) stain. Thus, prevention of UVB-induced cell death by Asc2P was shown to occur concurrently with inhibition of DNA cleavages and enrichment of intracellular Asc.

Keywords: *DNA strand cleavage, UVB-induced DNA lesions, apoptosis, ascorbic acid-2-O-phosphate, keratinocytes*

Introduction

A recent increase in ultraviolet B (UVB) ray reaching the earth's surface due to the progressive depletion of stratospheric ozone layer may induce a corresponding increase in skin cell death and skin cancer [1–4]. Recently, the effects of climate changes such as elevations in UV irradiance and temperature may certainly become some of the serious causes for skin cancer [5,6]. UV-induced cell death may be potently inhibited by administration of reactive oxygen species (ROS)-scavengers, among which L-ascorbic acid (Asc) is influentially nominated in terms of rapidity in scavenging of ROS existing in the water-soluble phase of the living body [7,8]. This is because, after incubation of human blood plasma with AAPH-derived aqueous peroxy radicals, lipoprotein lipids begin to undergo no hydroperoxidation until endogenous Asc is depleted earliest out of diverse ROS scavengers [9]. Administration of dehydroascorbic acid (DehAsc) that is an oxidized form of Asc, to mice, heightens the intracellular Asc concentration and prevents the ischemic cellular injury through quenching of ROS [10]. Asc prevents the myocardial mitochondrial dysfunctions such as decreases both in oxygen consumption and respiratory control ratio concurrently with depletion of mitochondrial glutathione, which are induced by post-ischemic reperfusion in the myocardium assumedly via ROS

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generation. UV-irradiation at lower-irradiances to hairless mice induced the depletion of endogenous Asc rapidly, but not other non-enzymatic antioxidants in the epidermis [11]. Such a phenomenon was observed also in the human plasma [12]. However, exogenous Asc has not been demonstrated to prevent UV-induced DNA lesions and resultant cell death assumedly owing to its lability in the aqueous solution.

In the present study, we therefore, examined preventive effects of Asc or its autooxidation-resistant derivatives on UVB-induced cellular damages such as DNA fragmentation, cell membrane disintegration and mitochondrial disfunction as the resultant cell death in mouse epidermal keratinocytes.

Materials and methods

Cell culture

Mouse epidermal keratinocytes Pam212 were a generous gift from Dr Toyoshi Fujimoto (Gunma Univ) [13]. They were cultured in DMEM (Nissui Pharm., Tokyo) supplemented with 10% fetal bovine serum (FBS) (GIBCO Oriental, Ltd, Tokyo).

UV irradiation and cell viability assay

Cells grown at the logarithmic phase were seeded at densities of $0.5-4 \times 10^4$ cells/well in a 24-well plate so as to proliferate up to the subconfluent state upon UV irradiation, and received, 18 h later, ascorbic acid (Asc; Sigma Chemical Co.), Asc-2-O-phosphate 2, 3 magnesium salt (Asc2P; Showa Denko Co., Tokyo) or dehydroAsc (Fluka) for 2 or 24 h. After aspiration, rinsing and feeding with phosphate buffered saline (PBS), cells were irradiated through a plastic cutoff-filter sheltering the slightly mingled UVC ray with or without a Spectronics UV transilluminator EBF-260 as a UVB ray resource. The irradiation was conducted under moistening conditions, with pouring PBS on the cell layer and the sterilized water into the well-surrounding space, so as to prevent the vaporizing-induced hypertension of the cell layer. UV irradiance was calibrated with an Ieda dosimeter UV-Response-Meter VLX-312. After irradiation, PBS was replaced by DMEM-10% FBS containing Asc/the derivatives or none, and further cultured for 24 h.

WST-1 cell viability assay

UVB-irradiated or control cells were further cultured for 24 h and then subjected to cell viability assay based on photometry for cellular ability to convert the redox indicator dye WST-1 (Cell counting kit, Dojin Chemicals, Kumamoto, Japan) into formazan primarily attributable to mitochondrial dehydrogenase activity. A 110 μl of WST-1 (8%) solution was added to the cell layer per a well. After incubation at 37°C for 1 h, the resultant diformazan formation was determined by measuring the absorption at 450 nm with a plate reader (Benchmark, Bio-Rad Laboratories, CA).

Trypan blue dye exclusion assay

The UVB-irradiated or control cells were further cultured for 24 h, then were suspended by trypsin, and received an equivoluminal freshly prepared trypan blue solution in MEM (0.20%). Microscopic counts of living (unstained) and dead (stained) cells were conducted on a hemocytometer.

ELISA assay for histone-associated DNA fragments

The extent of intracellular DNA strand cleavages was quantified with a cell death detection ELISA^{PLUS} kit (Boehringer Mannheim) that detects cytoplasmic histone-associated DNA fragments. A mixture of anti-histone-biotin and anti-DNA-POD antibodies was added to cell lysates in a streptavidin-coated microtiter plate. After the removal of unbound antibodies, the amount of DNA fragments was spectrophotometrically quantified by measuring POD activities at 405 nm.

DNA ladder assay

UV-irradiated or control cells were pelleted, lysed with 0.5% sodium *N*-lauroylsarcosinate and incubated with RNase A and subsequently Proteinase K. DNA was quantified with Hoechst 33258 and analyzed on 2% agarose/ethidium bromide gels (GIBCO-BRL). Electrophoretic pattern was assessed by densitometry using NIH Image software.

TUNEL method

DNA fragmentation of individual cells was detected *in situ* by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with the *in situ* Apoptosis Detection Kit (Takara, Kyoto). Cells grown on coverslips (2.4×10^4 cells in a 35 mm dish) were washed three times with PBS(−) and fixed with paraformaldehyde solution (3.7% in PBS(−)) for 15 min at room temperature. The cells were treated with 0.3% H_2O_2 in methanol for 20 min at room temperature to block endogenous peroxidase and washed three times with PBS(−). Then the cells were permeabilized in a solution containing 0.1% Triton X-100 for 5 min on ice, followed by incubation in freshly prepared TUNEL reaction mixture for 90 min at 37°C in the dark. The coverslips were washed with PBS(−) and mounted on slides with anti-fading solution. TUNEL staining was analyzed with a confocal laser fluorescence scanning microscope (Bio-Rad).

HPLC methods

To quantify intracellular ascorbic acid (Asc_{in}) contents, cells of a known number were made to receive freezing and thawing twice and were crushed with a Potter-type teflon homogenizer for 30 s on ice. The cell homogenate was centrifuged at 5°C and the supernatant thus separated was stored on ice. Cell debris pellet was suspended in PBS(−) and centrifuged again. The supernatant thus obtained was combined with the supernatant that was previously stored, and determined for the protein content with DC protein assay kit (Bio-Rad). Combined supernatants were filtrated with a Millipore filter Molcut II, and an aliquot of 50 μl was injected on an octadecylsilica gel-prepacked column Shodex ODSpak F-411A of 4.6 × 150 mm (Showa Denko that was connected in a series circuit of a Gilson HPLC system 305), followed by development with mobile phase solution consisting of 0.1 M sodium acetate, 0.02% EDTA and 0.017% *n*-octylamine at 40° and at a flow rate of 1.5 ml/min. Asc and Asc2P were detected with a coulometric electrochemical detector (ECD) Coulochem II (ESA Co., Bedford) at −200 mV/150 mV and/or with a Gilson UV detector 115 at 254/265 nm. Authentic Asc or Asc2P that was transiently mixed with the extract from control cells was similarly treated till injection on an HPLC column, and was detected as both a chromatographic peak area and a retention time similar to those of Asc or Asc2P dissolved in Milli-Q ultrapure water.

Results

Preventive effects of vitamin C derivatives on UVB-induced cell mortality of keratinocytes

Asc and its derivatives of 65 μM were administered for 2 h to the mouse keratinocytes Pam212, which were irradiated with UVB ray at an irradiance of 20 mJ/cm^2. After the subsequent 48 h cultivation, the rate of cell survival was measured as the cellular reducing activity principally due to mitochodrial dehydrogenase by formazan dye-based WST-1 method. It was demonstrated that the inhibitory effect on UVB-induced cell death was exerted with Asc or Asc-2-O-phosphate (Asc2P), but not exerted with Asc2G or DehAsc (Figure 1). To examine the superiority of Asc or Asc2P, the cytoprotective effects were similarly examined at its two addition doses against UVB of two irradiation doses. Asc2P, an autooxidation-resistant type of vitamin C, was shown to exceed Asc regardless of their administration doses or UVB irradiances examined (Figure 2). A slight enhancement of cell viability relative to the control was detected, without an increase in cell numbers, for Asc2P of 65 μM at a UVB dose of 10 mJ/cm^2, suggesting a possible hormesis-like mitochondrial activation as measured by WST-1 method.

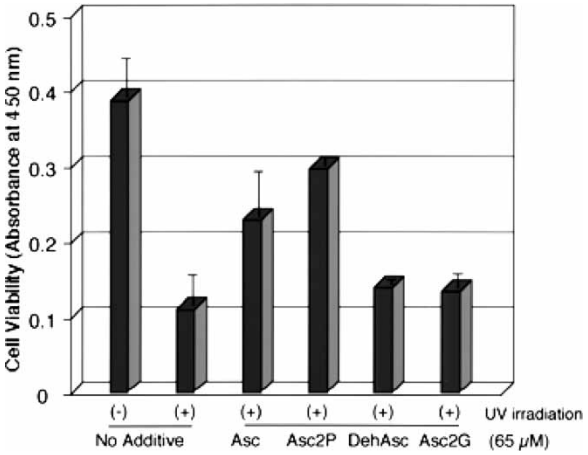


Figure 1. Preventive effects of Asc or its derivatives on UVB-induced cell mortality of mouse skin keratinocytes Pam212.Cells were previously administerd for 2 h with or without Asc derivatives such as DehAsc, Asc2P and Asc2G of 65 μM, and irradiated with UVB at 20 mJ/cm^2. After further 24 h cultivation, cell viability was assessed by photometric determination of mitochondrial dehydrogenase activity using the formazan-forming dye WST-1. These data shown are typical out of four independent experiments, and expressed as the averages of values of wells in triplicate, where the standard deviation (SD) is indicated by the vertical bar.

Dependence of the cytoprotective activity of the pro-vitamin C agent, Asc2P, on the administration modes

To determine the optimal mode of Asc2P administration, Asc2P at graded doses of 50, 100 or 200 μM was added at 2 or 24 h before UVB irradiation. The treatment of Pam212 keratinocytes with Asc2P for a period as long as 24 h was shown to be more cytoprotective than that of 2 h on all three administration doses and two UVB irradiation conditions (Figure 3(A)). This result showed that an administration period longer than 2 h was necessary for fully exhibiting the cytoprotective activity of Asc2P.

Further administration of fresh Asc2P after UVB irradiation besides its pre-irradiated administration ("throughout" in Figure 3(B)) was examined for its precipitative effects on the cytoprotection against UVB irradiation. However, there was no significant difference between the mode of pre-irradiated administration and that of "throughout" administration with Asc2P. This result suggests that Asc2P exerted no marked role in cellular post-irradiated repair for photodamages. No appreciable cytoprotection was achieved upon a UVB irradiance as high as 40 mJ/cm^2 except a mode of both pre- and post-irradiated administration ("throughout") with Asc2P as abundant as 200 μM. On the other hand, the significant cytoprotection was observed against

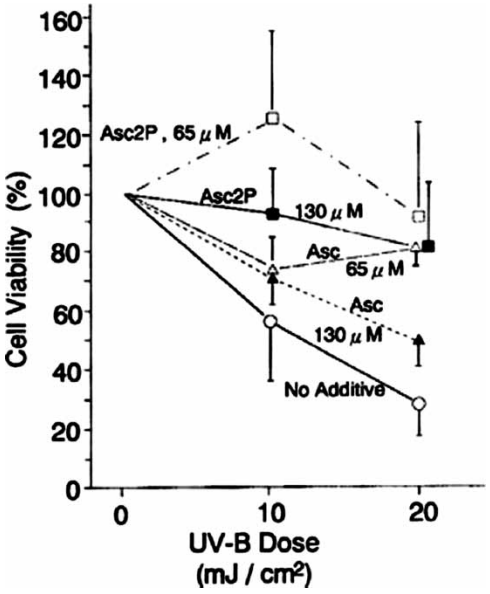


Figure 2. Cytoprotective effects of Asc or Asc2P against UVB-irradiation in Pam212 keratinocytes. Cells were previously administered with Asc or Asc2P of indicated doses, and irradiated with UVB at indicated doses, followed by assay for cell viability using WST-1 dye as conducted in Figure 1. The control cells were similarly conducted sham irradiation and administration with the culture medium containing neither Asc nor Asc2P. The data shown are typical out of three independent experiments, and expressed as the averages of values of wells in triplicate, where the SD is indicated by the vertical bar.

irradiation with UVB at irradiances as low as 10 or 20 mJ/cm^2 on any administration conditions. Thus the cytoprotection of Asc2P against UVB irradiation may be attributed to the preventive, but not therapeutic, effect of pre-irradiated administration with Asc2P, which was exerted to be limited within UVB irradiances lower than the definite level.

Evaluation for the cytoprotection against UVB irradiation by dye exclusion assay

To confirm the cytoprotective effects of Asc2P (Figures 1–3) by a method other than a redox indicator-based WST-1 assay that principally measures the mitochondrial dehydrogenase activity, a dye exclusion assay was applied using trypan blue. The UVB-irradiated Pam212 keratinocytes were separated into three categories such as cells that were detached from the culture dish and the attaching cells that were stained with trypan blue or not, the two formers of which can be regarded as to be dead. The UVB irradiation at 50 or 100 mJ/cm^2 generated the detached cells of a small proportion, which were considered to be subsequently cytolysed into smaller debris. The trypan blue dye exclusion assay showed that Asc2P at 130 μM exerted preventive effects on cell mortality in appearance even at irradiances as high as 50–100 mJ/cm^2 of UVB (Figure 4), where no

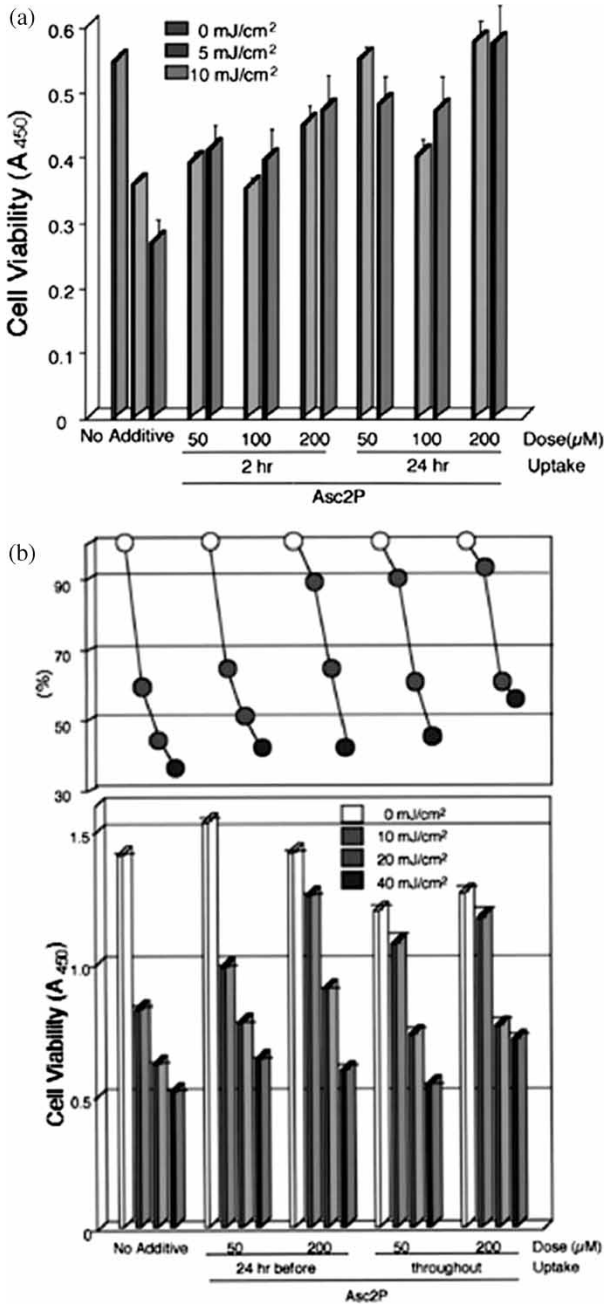


Figure 3. Cytoprotection by Asc2P against cell injuries induced by irradiation with UVB ray to Pam212 keratinocytes. (A) Cells were previously administered with or without Asc2P of indicated doses, and irradiated with UVB ray at indicated doses, followed by assessment of cell viability by WST-1 method. (B) The cytoprotective effects were determined in the same manner as done in Figure 3(A) except an administration mode of "throughout" that post-irradiated administration of fresh Asc2P at the same doses was supplementarily conducted besides the pre-irradiated administration. The data shown are typical out of three independent experiments, and expressed as the averages of values of wells in triplicate, where the SD is indicated by the vertical bar.

cytoprotection was achieved as estimated by WST-1 assay (Figure 3(B)). This result suggested that the trypan blue dye is a hypo-susceptible indicator for detection of UVB-induced cell mortality because the

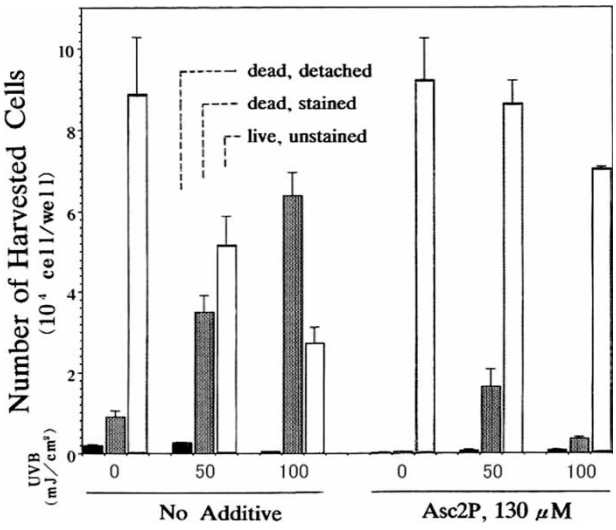


Figure 4. Preventive effects of Asc2P on induction of cellular injuries in UVB-irradiated Pam212 kerationocytes. Cells were previously administered with or without Asc2P of the indicated dose, and irradiated with UVB at indicated doses. The metabolic cell death and cell detachment from the culture substratum were then assessed by trypan blue dye exclusion assay and Coulter electric particle counter assay, respectively. The data shown are typical out of three independent experiments, and expressed as the averages of values of wells in duplicate, where the SD is indicated by the vertical bar.

dye was not incorporated into UVB-irradiated keratinocytes that were inactivated for mitochondrial dehydrogenase which is coupled with WST-1 assay used in Figures 1–3.

Involvement of diminished extents of DNA cleavages in the preventive effects of Asc2P against UVB-induced cell mortality

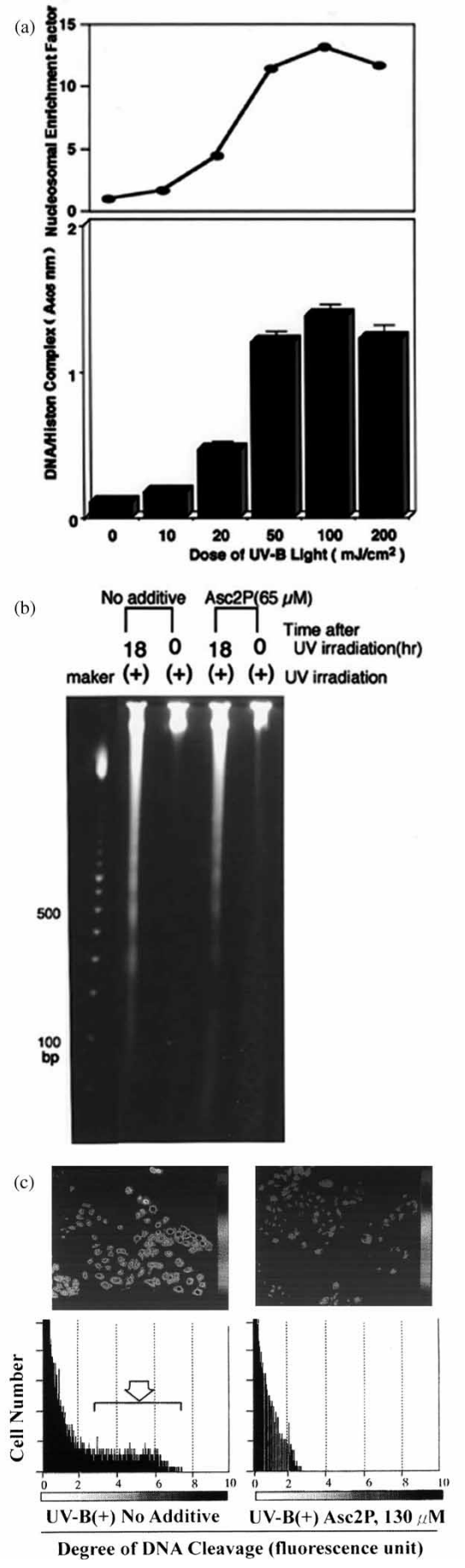
The DNA strand cleavages were quantified by ELISA method after UVB irradiation to Pam212 keratinocytes. As UVB irradiance was heightened, the yield of histone-bound DNA fragment complex was increased (Figure 5(A)). The generation of DNA fragments reached the maximum upon irradiation with UVB at 50 mJ/cm^2, with a plateau until 200 mJ/cm^2. In contrast, UVB irradiances where the appreciable cytoprotection with Asc2P was achieved, Figure 3(B) were restricted to 10–20 mJ/cm^2 Figure 5(A), where amounts of DNA/histone complex were as small as 3.9–25.2% versus the maximum amount at 100 mJ/cm^2, suggesting that ELISA method was difficult to demonstrate the marked cytoprotection. Thus the effect on degrees of DNA fragmentation as quantified by the ELISA method was suggested not to be explicitly responsible for the cytoprotection of Asc2P against UVB irradiation.

The DNA cleavages were detected as smears or ladders in the electrophoregrams of DNA extracts from UVB-irradiated Pam212 keratinocytes (Figure 5(B)). The UVB-induced DNA cleavages were detected by the electrophoretic smears or partly ladders at 300–800 bp for extract from cells that had not administrated Asc2P, whereas the extensive DNA cleavages were repressed as indicated by both slightly abundant intact DNA at an electrophoretic origin point (increased by about 17% as compared with that in non-administrated cell) and deletion of DNA fragments shorter below 500 bp by medication with Asc2P before UVB irradiation.

Estimation by the TUNEL method also demonstrated that UVB-induced DNA fragmentation was repressed notably by Asc2P, Figure 5(C). The dense TUNEL-staining was detected in the nuclei of UVB-irradiated cells more intensely (indigo-colored according to pseudo-coloring) than in the cytoplasm (cobalt-blue), where the DNA fragments that were primarily generated in the nuclei might leak out through the nuclear membrane. In contrast UVB-irradiated cells that received Asc2P before the irradiation represented no dense TUNEL-staining (yellowish). In concert with the distribution of DNA fragments, the histograms for the TUNEL staining of UVB-irradiated cells showed the high frequency of DNA-cleavage-abundant cells near the center of the abscissa, which were not detected for Asc2P-added cells, Figure 5(C).

Contents of Asc in Asc2P-administrated keratinocytes

The preventive effects against UVB-induced cell mortality (Figures 1–4) and DNA cleavages were demonstrated to be exerted by the pro-vitamin C agent, Asc2P. However, it cannot scavenge the UVB-derived ROS in the intact form. So intracellular contents of Asc, which might be produced from Asc2P, were quantified by HPLC separation and simultaneous detection with electrochemical coulometry and UV absorbance (Figure 6). Extracts from the Asc2P-administrated keratinocytes were shown to be accumulated larger amounts of Asc until 6–24 h gradually after Asc2P administration, but concurrently with no detection of Asc2P itself that can be less sensitively detected as measured with a spectrophotometer. This result suggested that Asc was released via dephosphorylation from Asc2P and functioned as an effecter molecule that could directly scavenge UVB-derived ROS. In contrast the keratinocytes that were similarly treated with Asc itself accumulated more abundant Asc than the Asc2P-treated keratinocytes did at 1 h after the administration for any doses examined and at 2 and 4 h for doses as low as 50 or 100 μM. Under the other examined conditions such as incubation periods as long as 6–24 h and administration concentrations as high as 100–200 μM, however, superiority of Asc2P administration to Asc administration was always achieved in terms of intracellular Asc accumulation.



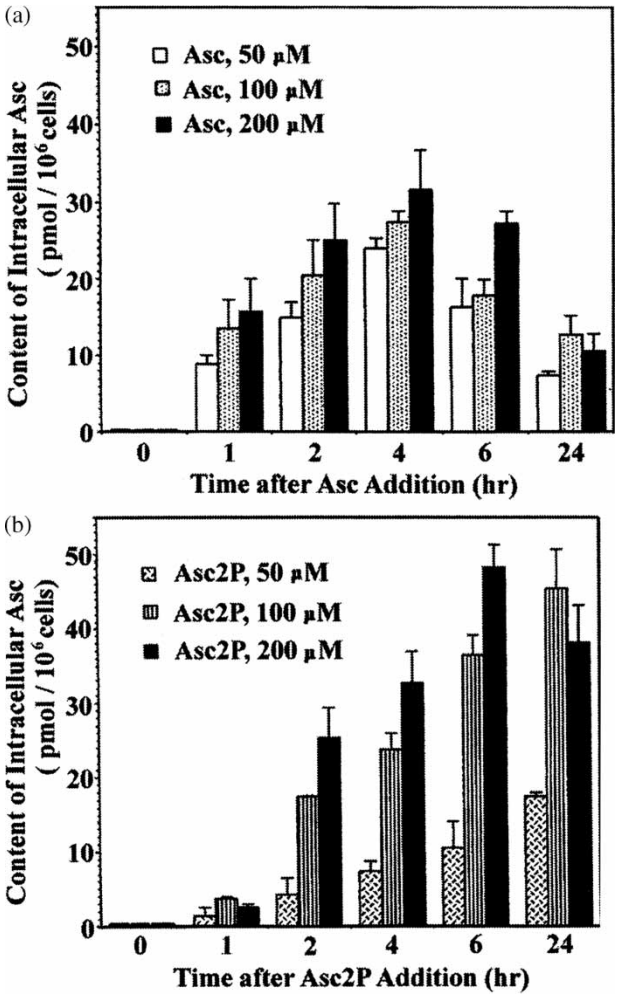


Figure 6. Intracellular Asc content in Pam212 keratinocytes that were administered with Asc (A) or Asc2P (B) as assessed by ion-paired HPLC separation and detection with a coulometric ECD and UV absorbance detector.

Figure 5. DNA strand cleavages in UVB-irradiated Pam212 keratinocytes and the repressive effects of Asc2P. (A) Dependence on UV irradiance of histone-bound DNA fragment complex expression in UVB-irradiated Pam212 keratinocytes. Cells were irradiated with UVB of graded doses, and after cultivation for 18 h, were assessed for the yield of histone/DNA fragment complex by the ELISA method. The data shown are typical out of two independent experiments, and expressed as the averages of values of wells in duplicate, where the SD is indicated by the vertical bar. (B) Pam212 cells were previously administered with or without Asc2P of 65 μM for 24 h, and irradiated with UVB at 15 mJ/cm^2. DNA was extracted from cells cultivated for 0, 18 h after UVB irradiation, and assessed for double strand cleavages by agarose gel electrophoresis. The data shown are typical out of six independent experiments. (C) Pam212 keratinocytes were administered with or without Asc2P of 130 μM for 24 h, and irradiated with UVB at 15 mJ/cm^2. The UVB-irradiated cells were further cultivated for 9 h, and were *in situ* detected for DNA double strand cleavages by TUNEL fluorescence method and histographic processing for fluorescent intensity. The data shown are typical out of five independent experiments.

Discussion

The preventive effect against UVB-induced cell mortality in mouse skin keratinocytes Pam212 was shown to be exerted by administration of Asc, and to be more appreciably exerted by the pro-vitamin C agent, Asc2P (Figure 1). This is because the exogenously administered Asc is so labile to auto-oxidation [14,15] as to be difficult to undergo the efficient uptake into intracellular space via oxidative conversion to DehAsc which is prone to be irreversibly transformed into diketogulonic acid [16,17]. This oxidized form of vitamin C, DehAsc, was, therefore, shown to be inactive for a cytoprotective effect against UVB irradiation (Figure 1). In contrast Asc2P is an autooxidation-resistant type of vitamin C which is protectively blocked at its 2, 3-enediol moiety [18,19] which would be easily subjected to auto-oxidation if being in the unblocked form. More marked cyto-protection by Asc2P than Asc itself is attributed to both the gradual release of Asc from Asc2P and subsequently its efficient uptake into keratinocytes as demonstrated by HPLC analysis for intracellular Asc contents (Figure 6). No preventive effect of Asc2G on UVB-induced cell mortality (Figure 1) is considered to be attributed to both its extreme stability [20,21] during contact to the keratinocytes that is insufficient for alpha-glucosidase and the resultant inefficient conversion to Asc like another pro-vitamin C agent, Asc-2-O-sulfate [22,23].

The optimal cytoprotection with Asc2P against UVB irradiation was shown to necessitate a pre-irradiated incubation with Asc2P for a period as long as 24 h preferentially over 2 h, Figure 3(A). This need of longer-period preincubation of Asc2P for cytoprotection is consistent with more abundant contents of intracellular Asc according to longer-period administration with Asc2P (Figure 6). In addition, post-irradiated supplimentary administration of Asc2P was shown not to further enhance the cytoprotective effects of pre-irradiated incubation with Asc2P, Figure 3(B). As combined with these results, previous accumulation of intracellular Asc until a timing of UVB irradiation was suggested to be responsible for the cytoprotection by Asc2P against UVB, and to be achieved by pre-irradiated incubation for a period longer than 2 h necessary for conversion via dephosphorylation of Asc2P into Asc, which can scavenge ROS that is generated immediately after UVB irradiation [9,24–27], but Asc cannot repair the post-irradiated cell injuries. Upon 2 h administration, Asc-administration achieved an intracellular-Asc accumulation similar to that by Asc2P-administration (Figure 6), but achieved a cytoprotection slightly inferior to that of Asc2P-administration (Figures 1 and 2). This inconsistency in appearance may be attributed to a difference in stability of extracellular Asc and Asc2P against oxidation induced by UVB irradiation, in contrast to the experiment on intracellular Asc uptake that was conducted in the absence of UVB irradiation (Figure 6). The cytoprotective ability of Asc2P was shown to be restricted within irradiation with UVB at doses below 5–20 mJ/cm^2, but not to be exhibited over UVB doses as high as 40 mJ/cm^2, Figure 3(B), which nearly equals the minimum erythema dose for human skin [28–32] and might generate explosively such a great deal of ROS as could not be overcome even by the maximum accumulation of intracellular Asc. It should, therefore,be strictly avoided to be exposed to UVB irradiation as intense as generating erythema partly because of taking it into consideration that Asc2P as a putative skin-protector is not almighty.

The cytoprotective effects of Asc2P against UVB were demonstrated in terms of three cell-viability indicators such as mitochondrial dehydrogenase activity as the cell survival rate (Figures 1–3), the exogenous dye-excluding ability (Figure 4) and DNA strand cleavage, Figures 5(B),(C). The UVB-induced cleavages of DNA strands are considered to be attributed to both the radiation effect [33–36] and the oxidative effect of UVB ray [37,38], the latter of which is executed via amplification with peri-nuclear photo-sensitizers [39–41] including porphirins and riboflavins, and can be prevented by intracellular antioxidants such as Asc. The radiation effect via no intervening mediator cannot be counteracted by antioxidants, and may contribute more markedly to UVB-induced cell mortality above 40 mJ/cm^2 of a UVB dose, Figure 3(B). The time-dependent increase in intracellular Asc after Asc2P-administration to the keratinocytes (Figure 6) suggested that Asc was esterolytically released from Asc2P assumedly through extracellular or membrane-bound phosphatases, and entered into the intracellular space via Asc transporter proteins such as SVCT-1, 2 [42–45] that are located on the cell surface. A great deal of Asc accumulated thus in the cytoplasm may be migrated efficiently into the nucleus through the nuclear channels, composed of nucleoporin, known to permit the bi-directional exchange of soluble molecules smaller than 5000 Da [46–48] such as Asc. Existence of abundant Asc in the vicinity of DNA or chromatins can be substantiated by a steep transmembrane gradient of Asc concentrations between the nucleus and the cytoplasm compartments. The UVB-generated ROS near DNA strand can be efficiently scavenged by the neighboring Asc before formation of DNA injuries due to the UVB-induced oxidative effect.

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