Ascorbic Acid 2-\(\alpha\)-Glucoside, a Stable Form of Ascorbic Acid, Rescues Human Keratinocyte Cell Line, SCC, from Cytotoxicity of Ultraviolet Light B

Eriko Miyai, Mitsuhiko Yanagida, Jun-ichi Akiyama, and Itaru Yamamoto

Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Okayama 700, Japan, and Department of Research & Development, Kaminomoto Co., Ltd., 3-3-25 Kamocho-bashi-dori, Chuo-ku, Kobe 651, Japan. Received November 29, 1995; accepted March 1, 1996.

Environmental exposure to ultraviolet light B (UVB, wave lengths 290—320 nm) of the solar spectrum causes major damage, including an inflammatory response, in skin. In the present study, we estimated the ability of a stable derivative of ascorbic acid, ascorbic acid 2-\(\alpha\)-glucoside (AA-2G), to reduce UVB damage, using the human keratinocyte cell line, SCC, established from squamous cell carcinoma. By pre- (9h) and post-cultivation with AA-2G, a significant preventive effect on the decrease in the absolute number of surviving cells by exposure to UVB (typical dose, 20 mJ/cm\(^2\)) was measurable by a neutral red-uptake assay. The release of lactate dehydrogenase from the cell membrane damaged by UVB was inhibited by AA-2G. In agarose gel electrophoresis, relatively high molecular weight DNA fragments were detected in irradiated cells after 6 h post-irradiation, suggesting that the mechanism of cell death was necrosis. Quantitative analysis of DNA content by flow cytometry indicated that AA-2G suppressed both an increase in debris with degraded nuclei and a decrease in cells in G1 and S phases, but not in the G2/M phase, by UVB exposure. These data suggest that AA-2G shows a photoprotective effect against UVB-induced damage in human epithelial cells.

**Key words** ascorbic acid 2-\(\alpha\)-glucoside; UVB; photoprotective effect; human keratinocyte cell line; SCC

Ultraviolet light B (UVB), which consists in wave-lengths between 290 and 320 nm, is a major cause of morbidity to human skin, both acutely (sunburn) and chronically (photaging and skin cancer). A number of studies demonstrated that reactive oxygen species and lipid peroxides are among the most reasonable candidates for explaining UVB injury. To diminish the influence of UVB damage, the regulation of antioxidant capacity in skin has been examined, which included the maintenance of adequate levels and the localization of antioxidant compounds and enzymes. An effective defense against actinic injury, however, may require not only balanced doses of antioxidant enzymes (superoxide dismutase, catalase, etc.) but also an improved drug delivery system.

Based on these points, several low molecular weight antioxidants are now the subject of experimental trials. It has been considered that ascorbic acid (AsA) plays a role in the prevention of UV-induced oxidative damage in skin due to its strong reducing activity. Chakraborty et al. have described the protective effect of AsA against lipid peroxidation of guinea pig tissues in *vivo* and in *vitro*. In fact, daily pretreatment using AsA from 3 d to 1 week provided protection of the skin from UVB damage as measured by erythema and sunburn cell formation. It is, however, difficult to develop AsA as an anti-inflammatory agent due to its rapid degradation; therefore, its chemical modification is necessary for a prolonged half-lifetime.

We have recently synthesized a new derivative of AsA, ascorbic acid 2-\(\alpha\)-glucoside (AA-2G), which is highly stable under various oxidative conditions. AA-2G is hydrolyzed to AsA by \(\alpha\)-glucosidase in mammalian cells and tissues to express the reducibility. In researching the biological effects of AA-2G as a precursor of AsA, we have reported prevention against hydrocortisone-induced cataract formation in developing chick embryos, which is caused by cellular oxidative damage by hydrocortisone. A broader specificity of AsA for protection from oxidative stress is considered to exist in various tissues, whereby it might be possible that AA-2G could reduce UV light injury to skin.

In this study, we investigated the ability of AA-2G to induce resistance against UVB cell injury, using human the keratinocyte cell line, SCC, established from squamous cell carcinoma. We reported that this derivative of ascorbic acid could diminish the lethal effect of UVB exposure in keratinocytes in *vitro*.

**MATERIALS AND METHODS**

**Materials** The material sources used in this work were as follows: AA-2G from Hayashibara Biochemical Labs. (Okayama, Japan), sodium pyruvate and Gansa solution from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Neutral Red Assay Kit from Kurabo Co., Ltd. (Osaka, Japan), Dulbecco’s minimum essential medium (DMEM) and \(\gamma\)-glutamine from Nissui Pharmaceutical Co. (Tokyo, Japan), fetal bovine serum (FBS) from International Reagents Co. (Kobe, Japan), \(\alpha\)-nicotinamide adenine dinucleotide, reduced form (NADH), ethidium bromide, propidium iodide (PI) and ribonuclease A (RNase A, Type III-A) from Sigma Co., Ltd. (St. Louis, MO, U.S.A.), agarose from Takara Shuzo Co., Ltd. (Shiga, Japan) and *Hind*III digest of \(\lambda\)-DNA from Toyobo Co., Ltd. (Tokyo, Japan).

**Cell Culture and Cytotoxicity Test** SCC cells were kindly provided by the Department of Dermatology, Shinshu University School of Medicine. The cells were plated on 24-well dishes (Falcon) at 2.5 x 10^4/Well in a growth medium consisting of DMEM supplemented with 4.0 mm \(\gamma\)-glutamine and 10% FBS. After 3 d, the cells...
were preincubated with various concentrations of AA-2G for 9 h, then the medium was replaced in phosphate buffered saline (PBS) containing 0.90 mM CaCl₂ and 0.49 mM MgSO₄ during exposure to UVB. UVB irradiation (5–40 mJ/cm²) was carried out for 80 s with a bank of 4 closely grouped FL20S-E lamps (Toshiba Electric Co., Ltd., Tokyo, Japan) as a light source. The intensity of UVB was fixed at 0.250 mW/cm² by measurement with a UV radiometer (model UVR-305/365-DII, Tokyo Kagaku Kiki Co., Ltd., Tokyo, Japan) in all of the experiments. After irradiation, cells were incubated in growth medium containing various concentrations of AA-2G for 24 h. A Neutral Red Assay Kit was used to estimate cytotoxicity. Briefly, the cells were incubated with 50 μg/ml neutral red (NR) for 2 h and then fixed in 1% (v/v) formalin containing 1% (w/v) CaCl₂ for 1 min. The pigment incorporated into intracellular lysosomes was extracted in 300 μl of 0.4% HCl containing 2-propanol, and the absorption level at 540 nm was measured in a NP-500 microplate reader (Kurabo). The number of viable cells was calculated by the standard curve between optical density and cell number, which was correlated in the range from 0.7 × 10⁶ to 14.3 × 10⁶ cells/well.

Measurement of Lactate Dehydrogenase (LDH) Activity At the end of the incubation period of the cytotoxicity test (at 24 h after UVB irradiation), LDH activity in the culture supernatants was measured as described elsewhere. B Briefly, 0.1 ml of a sample medium was incubated with 2 ml of 0.476 mM sodium pyruvate in 0.05 M KH₂PO₄–K₂HPO₄ buffer (pH 7.4) and 0.1 ml of 2 mM NADH at 28°C, and the change in absorbance was immediately monitored at 340 nm.

DNA Gel Electrophoresis SCC cells grown for 3 d in a 60 mm dish (Falcon) (4 × 10⁵/dish at inoculation) were exposed to UVB (20 mJ/cm²). Before and at appropriate times after irradiation, cells were subjected to DNA gel electrophoresis and DNA-flow cytometry. The cells harvested by trypsinization were suspended in 100 μl hypotonic lysis buffer (0.2% Triton X-100, 10 mm Tris and 1 mm EDTA, pH 8.0) and centrifuged at 13800 × g for 10 min. The supernatant was separated immediately and incubated with 4 μl RNase A (10 mg/ml) for 30 min at 37°C. After the addition of 100 μl proteinase K (0.4 mg/ml) in lysis buffer, the mixture was incubated for 30 min at 37°C and mixed with 22 μl 5 m NaCl and 222 μl absolute 2-propanol. Samples were allowed to stand at −20°C overnight and centrifuged at 13800 × g for 20 min. The pellet was washed with 200 μl 70% ethanol, then allowed to dry at room temperature. DNA was resuspended with 12 μl Tris–EDTA buffer (10 mm Tris–HCl, 1 mm EDTA, pH 8.0) and 3 μl loading buffer (50% glycerol, 10% saturated bromophenol blue and 1% xylene cyanol in 40 mm Tris–HCl, 19.7 mm Na acetate, 1 mm EDTA, pH 7.2), incubated at 37°C for 20 min, then electrophoresed on 1.5% agarose gel containing 0.71 μg/ml ethidium bromide for 1 h. The gel was photographed using a UV transilluminator.

DNA-Flow Cytometry Analysis The DNA content of SCC cells was determined by flow cytometry (FCM) analysis according to the modified method described by Gong J. et al. At 17 h post-irradiation, cells were kept in 5% FBS–PBS on ice for 30 min and gently isolated by trypsinization. Two million cells were fixed in 70% ice-cold ethyl alcohol at 4°C overnight. After being washed with PBS once, they were suspended in 0.1 ml of phosphate-citrate buffer consisting of 192 parts of 0.2 M NaPO₄ and 8 parts of 0.1 M citric acid (pH 7.8), at room temperature for 30 min. Then, they were processed with 0.25 mg/ml RNase A at 37°C for 30 min, stained with 50 μg/ml PI at 4°C for 30 min and filtered through a nylon mesh. Within 30 min, the level of nuclear DNA in 2 × 10⁶ cells was determined using an Epics Profile flow cytometer (Coulter Electronics, Hialea, FL). The data obtained with FCM were analyzed using the Multicycle program (Phoenix Flow Systems, San Diego, CA) to determine the percentages of debris with degraded nuclei and of cells in G1, S and G2/M phases.

Statistical Analysis Means and standard deviations were compared by Student’s t-test with significant probability levels of < 0.05.

RESULTS AND DISCUSSION

![Graph A](image1.png)

**Fig. 1. Effect of AA-2G on Survival of SCC Cells Exposed to UVB**

A, SCC cells pretreated for 9 h with (■) or without (□) AA-2G (1 mM) were exposed to UVB (5–40 mJ/cm²) and recultured for 24 h in the same conditions at precubation. Then, the number of viable cells was measured by NR assay. Data are expressed as means ± S.D. of quadruplicate cultures. *p < 0.05, **p < 0.01 compared to the corresponding control cultures. B, SCC cells pretreated for 0–24 h with AA-2G (1 mM) were exposed to UVB (20 mJ/cm²) and recultured for 24 h in the presence (●) or absence (○) of AA-2G. The number of viable cells are expressed as means ± S.D. of quadruplicate cultures.
cytotoxicity using the NR assay. As shown in Fig. 1A, UVB showed a lethal effect on SCC cells in a dose-dependent manner. Pre- (9 h) and post-cultivation with AA-2G (1 mm) afforded a significant prevention of the decrease in viable cell number at 24 h after irradiation (10—40 mJ/cm²) (Fig. 1A). This protection by AA-2G was more effective than that by ascorbic acid (data not shown). Preincubation for 9 h of the cells in the presence of AA-2G was enough to protect against UVB-induced cell injury (Fig. 1B). The inhibitory effect of AA-2G against UVB-induced cytotoxicity was dose-dependent at a range of concentrations from 0.1 to 1 mm (Table 1). As

<table>
<thead>
<tr>
<th>Irradiation of UVB</th>
<th>AA-2G (µM)</th>
<th>Viable cell number x 10⁶ cells/well</th>
<th>LDH activity in culture supernatant A440/min/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unirradiated cells</td>
<td>0</td>
<td>11.12 ± 0.22</td>
<td>0.096 ± 0.013</td>
</tr>
<tr>
<td>Irradiated cells</td>
<td>0</td>
<td>5.25 ± 0.46</td>
<td>0.358 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>7.05 ± 0.39*</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>8.09 ± 0.28**</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.23 ± 0.45**</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>9.67 ± 0.13***</td>
<td>0.221 ± 0.018**</td>
</tr>
</tbody>
</table>

SCC cells pretreated for 9 h with AA-2G (0.1—1.0 mm) were exposed to UVB (20 mJ/cm²) and recultured in a culture medium supplemented with AA-2G for 24 h. Then, the number of viable cells was measured by NR assay. At the same time, the culture supernatant was removed from each well and its LDH activity was determined colorimetrically at 340 nm. Data are expressed as means ± S.D. of quadruplicate cultures. *p < 0.01, **p < 0.001, ***p < 0.0001 compared with the UVB-irradiated control cultures. N.D.; not done.

Fig. 2. Detection of Degraded DNA in UVB-Irradiated Cells
SCC cells were harvested before (lane 2) or at 6 h (lane 3), 12 h (lane 4) or 24 h (lane 5) after irradiation (20 mJ/cm²). The DNA fragments isolated from lysed cells were electrophoresed on agarose gel. A HindIII digest of λ-DNA was run as a molecular size marker (lane 1).

Fig. 3. DNA-FCM Histograms and Cell Cycle Phase Distributions of SCC Cells after UVB Irradiation with or without AA-2G Treatment
SCC cells exposed to UVB (20 mJ/cm²) (conditions identical to those in Fig. 2) were harvested at 17 h and fixed. After treatment with RNase A, they were stained by propidium iodide and subjected to FCM. A—C, DNA-FCM histograms of cells before irradiation (A), irradiated control cells (B) or irradiated cells with AA-2G (1 mm) treatment (C). The debris area was indicated by a slashed region, and analyzed by the Multicycle program. D, the viability and results of DNA-FCM analysis (the percentage of debris and cell cycle phase distribution) of SCC cells indicated in A—C. The viability was measured by a trypan-blue exclusion test and DNA-FCM analysis was carried out by the Multicycle program. Data are expressed as means of duplicated cultures in a representative result. The changes of percentages by irradiation are indicated in parentheses.
also shown in Table 1, AA-2G suppressed the release of LDH, a marker enzyme for cell membrane destruction, to the culture medium. These data suggest that AA-2G protects the cells from UVB-induced cell death rather than enhancing cell division. Indeed, AA-2G showed no effect on SCC cell proliferation without UVB irradiation (data not shown).

Martin and Cotter\textsuperscript{19} reported that human leukemia HL-60 cells rapidly undergo apoptosis en masse after a short period of UV irradiation, whereas prolonged exposure of these cells induced an other form of cell death which was suggestive of necrosis. We next tried to characterize a pattern of DNA degradation by UVB irradiation in this culture system and to detect cells with degraded nuclei. At 6 h post-irradiation, fragmented but relatively high molecular weight DNA appeared in a smearing pattern, suggesting necrotic cell death in agarose gel electrophoresis (Fig. 2). Likewise, the same smearing patterns of DNA were obtained at 12 and 24 h. With this experimental condition, we confirmed the characteristic ladder pattern of DNA from murine thymocytes, which reflects an apoptosis, as previously reported\textsuperscript{15} (data not shown). Figures 3A—C show DNA-FCM histograms of SCC cells before (A) and after irradiation without (B) or with (C) AA-2G treatment. Exposure of cells to UVB resulted in an increase in debris, indicated by a slashed region (Fig. 3B), in comparison with the histogram of intact cells (Fig. 3A). We confirmed that the UVB dose-dependent increase in debris with degraded nuclei (7.4, 10.3, 18.8, 36.9 and 50.2\% at 0, 2, 5, 10 and 20 mL/cm\textsuperscript{2}, respectively, analysed by the Multicycle program) reflected the lethal damage which was reduced by 1 mm AA-2G (Fig. 3C). Treatment with AA-2G (1 mm) decreased the percentage of debris and, in contrast, increased the viability measured by the trypan blue exclusion test (Fig. 3D). Kastan et al.\textsuperscript{20} reported that G\textsubscript{2} irradiation of human myeloblastic leukemia cells, ML-1, resulted in the G1 and G2 arrests. Thus, we tried to examine a cell cycle distribution of SCC cells after UVB irradiation. As shown in Fig. 3D, the percentages of SCC cells present in any phase were decreased by UVB irradiation. AA-2G, however, inhibited the UVB-induced decrease in the percentages of surviving cells in the G1 and S phases, but not in the G2/M phases. These data shown in this paper demonstrate that AA-2G could reduce the cell damage caused by UVB, which triggered SCC cells to undergo necrosis. Necrosis is known to result from an injury caused by various forms of lethal damage and to involve inflammatory reactions.\textsuperscript{21} Therefore, it is of interest to examine the effect of AA-2G on UVB-induced erythema in skin. Our ongoing studies have shown the preventive effect of AA-2G on UVB-induced erythema in human and guinea pig skin (unpublished data). More work will be required to elucidate the protective mechanism(s) of AA-2G against UVB injury, but it is clear that AA-2G is capable of allaying some of the deleterious effects of UVB upon cultured epithelial cells.

REFERENCES