Induction of gene expression via activator protein-1 in the ascorbate protection against UV-induced damage

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UV irradiation is a major insult to the skin. We have shown previously that exogenous vitamin C (ascorbate) accumulates in HaCaT keratinocytes, thus conferring the ability to prevent radical formation and cell death elicited by UV-B. Here, we have investigated the potential mechanisms accounting for the cytoprotective effects exerted by this antioxidant. Using a cDNA microarray hybridization, we identified several genes whose expression was up-regulated by ascorbate. We focused on the *fra-1* gene, a member of the Fos family of transcription factors that down-regulates activator protein-1 (AP-1) target genes. Both in HaCaT and in normal human epidermal keratinocytes, we found Fra-1 mRNA induction as early as 2 h after ascorbate loading. Electrophoretic mobility-shift assay and antibody supershift analysis revealed that ascorbate modulates AP-1 DNA-binding activity and that Fra-1 is in AP-1 complexes in treated cells. Furthermore, transient-transfection studies, using an AP-1 reporter construct, showed that ascorbate was able to inhibit both basal and UV-B-induced AP-1-dependent transcription. Ascorbate also modulates UV-B-induced AP-1 activity by preventing the phosphorylation and activation of the upstream c-Jun N-terminal kinase (JNK), thus inhibiting phosphorylation of the endogenous c-Jun protein. These data suggest that ascorbate mediates cellular responses aimed at counteracting UV-mediated cell damage and cell death by interfering at multiple levels with the activity of the JNK/AP-1 pathway and modulating the expression of AP-1-regulated genes.

Key words: Fra-1, JNK, vitamin C.

INTRODUCTION

Mammalian skin keratinocytes exposed to solar UV radiation exhibit a complex alteration of gene expression, which results in cutaneous inflammation, premature aging, tumour promotion and cell death [1–3]. In particular, UV-B (wavelength, 290–320 nm) has been shown to be the most relevant component of sunlight, acting as an initiator and promoter of skin cancer; UV-A (320–380 nm) and UV-C (100–290 nm) also might contribute to skin malignancy [4]. The skin-damaging effects of UV radiation mainly occur through the generation of free radicals; reactive-oxygen-species-mediated oxidative damage involves a vast number of biological molecules as it causes DNA modification [5], lipid peroxidation [6], surface expression of lectins [7] and secretion of inflammatory cytokines [8]. Moreover, environmental exposure of human keratinocytes to UV radiation results in an altered expression of several genes that are modulated in a dose- and time-dependent manner [9–13]; however, the molecular mechanisms involved have not yet been fully elucidated.

Mammalian cells have developed complex antioxidant-defence mechanisms to deal with oxidative stress promoted by UV radiation. In particular, ascorbate, the major water-soluble antioxidant found in extracellular and intracellular aqueous compartments, rescues keratinocytes from UV-mediated cytotoxicity directly scavenging radical species. Ascorbate has been shown to act as a general protective agent against cell death triggered by different stimuli and the cytoprotective effects have been attributed to the antioxidant properties of the vitamin. Ascorbate inhibits cell death induced by oxidative stress in a myeloid leukaemia cell line [14]; it also protects against apoptosis of thymocytes [15] and it can interfere with at least two different pathways (growth-factor withdrawal or activation with steroids) that promote apoptosis in T-cells [16]. Although the key role played by ascorbate and other antioxidants in photoprotection has been shown clearly, the exact mechanisms by which these molecules counteract UV-B-mediated damaging effects are not completely elucidated and remain at times controversial. Both direct (through antioxidant properties) and indirect (through transcriptional or post-transcriptional regulation) mechanisms have been implicated to explain the cellular sensitivity or resistance to UV-B radiation [17]. Ascorbate-supplemented keratinocytes showed a marked decrease in cell death elicited by UV irradiation and this protection was associated with inhibition of lipid peroxidation as well as with transcriptional down-regulation of interleukin-1α mRNA [18]. Furthermore, recent experimental data have pointed out the ability of ascorbate (and other dietary antioxidants) to modulate gene expression at the transcriptional level in several cell types, including the down-regulation of the matrix metalloproteinase-2 mRNA in cultured human amnion cells [19], the positive regulation of the tyrosine hydroxylase gene in the human neuroblastoma SK-N-SH cell line [20] and the prevention of radiation- [21] or lipopolysaccharide-induced [22] cell death through regulation of apoptosis-associated genes.

Oxidative stress elicited by UV irradiation activates redox-sensitive transcription factors, including nuclear factor-κB

Abbreviations used: AP-1, activator protein-1; AA-2P, L-ascorbate 2-phosphate; NHEK, normal human epidermal keratinocyte; JNK, c-Jun N-terminal kinase; CAT, chloramphenicol acetyltransferase.

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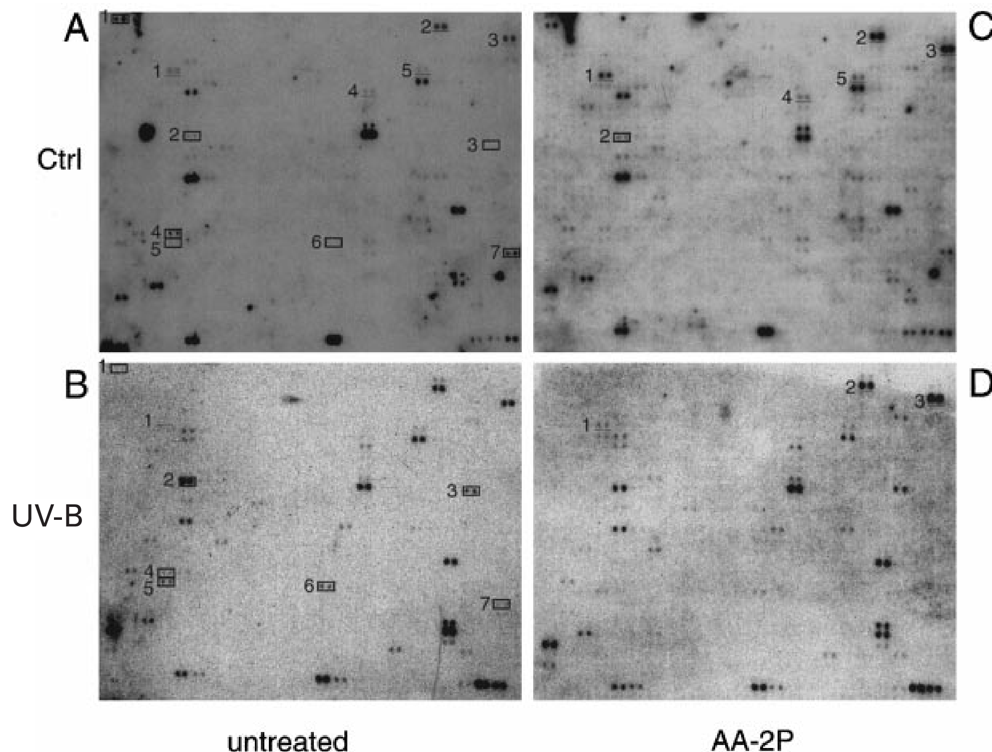

Figure 1 Analysis of expression of 588 transcripts using the Atlas cDNA Expression Array

^{32}P-Labelled cDNAs were obtained from poly(A)$^+$ RNAs isolated from HaCaT cells subjected to different treatments; the cDNA probes were then hybridized to the Atlas membranes according to the manufacturer's instructions (see the Experimental procedures section). (**A**) Untreated keratinocytes; (**B**) UV-B-treated (77 mJ/cm^2 for 1 min) cells; (**C**) AA-2P-loaded (1 mM for 5 h) cells; (**D**) irradiated keratinocytes in the presence of ascorbate. In each panel are outlined only those genes mentioned in the text. Transcripts regulated by UV-B (**B** versus **A**): up-regulation (S19 ribosomal protein, square 2; replication factor C, 38 kDa subunit, square 3; *ETR101*, square 5; α-catenin, square 6) and down-regulation [c-*myc*, square 1; *fra-1*, line 1; *ETR103* (*EGR1*), square 4; acyl-CoA-binding protein, square 7]. Transcripts up-regulated by AA-2P (**C** versus **A**): *fra-1*, line 1; *GST-pi*, line 2; *MLH1*, line 3; S19 ribosomal protein, square 2; natural killer cell-enhancing factor B (thioredoxin-dependent peroxidase 1), line 4; defender against cell death 1 (DAD-1), line 5. Autoradiography is representative of two different hybridizations from two different experiments; only modulations observed in both experiments are reported.

[23–25] and members of the activator protein-1 (AP-1) complex, such as c-Fos and c-Jun [26]. The activation of AP-1 results in the expression of AP-1 downstream target genes, such as γ-glutamylcysteine synthetase, glutathione S-transferase and quinone reductase [27–29].

In this study, we investigated the cytoprotective effects of vitamin C against UV-mediated cell damage at the level of gene expression. We have shown previously [30] that L-ascorbate 2-phosphate (AA-2P) is taken up from the extracellular medium and accumulated as ascorbate [31]. This compound is stable in the culture medium, thus avoiding the generation of oxidation and degradation products. In our model, preincubation of HaCaT keratinocytes with 50 μM AA-2P was enough to prevent radical formation and cell death induced by UV-B exposure [30]. To elucidate the molecular mechanism(s) accounting for the protection exerted by this antioxidant, we have used a cDNA microarray hybridization approach. We obtained an expression profile of genes associated with UV irradiation and the comparative analysis of irradiated cells, in the presence or absence of AA-2P, led us to identify *fra-1* as an ascorbate-responsive gene. We have also demonstrated that, in both transformed (HaCaT) and normal human epidermal keratinocytes (NHEKs), ascorbate was able to inhibit the AP-1-dependent transactivation of specific promoters by (i) modulating *fra-1* expression and (ii) preventing the phosphorylation of the c-Jun N-terminal kinase (JNK), which in turn phosphorylates and activates the c-Jun protein. In conclusion, ascorbate is a negative regulator of AP-1 and AP-1-dependent transcription, providing a novel transcriptional mechanism for ascorbate in mediating photoprotection.

EXPERIMENTAL

Reagents

Ham's F-12 and minimal essential medium were from Gibco (Berlin, Germany) and foetal calf serum was from HyClone (Oud-Beijerland, The Netherlands). Magnesium AA-2P was purchased from Wako Pure Chemical Industries (Neuss, Germany). RNase-free DNase I was obtained from Boehringer-Mannheim (Mannheim, Germany). Anti-JNK1 (C17), anti-phospho-JNK (G7) and anti-c-Jun (H79) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); anti-phospho-c-Jun (Ser 63) was purchased from New England Biolabs (Beverly, MA, U.S.A.). All reagents, unless otherwise indicated, were from Sigma (St. Louis, MO, U.S.A.).

Cell cultures

HaCaT cells, a spontaneously immortalized human keratinocyte cell line [32], were a generous gift from Professor N. E. Fusenig (Division of Differentiation and Carcinogenesis In Vitro, Institute

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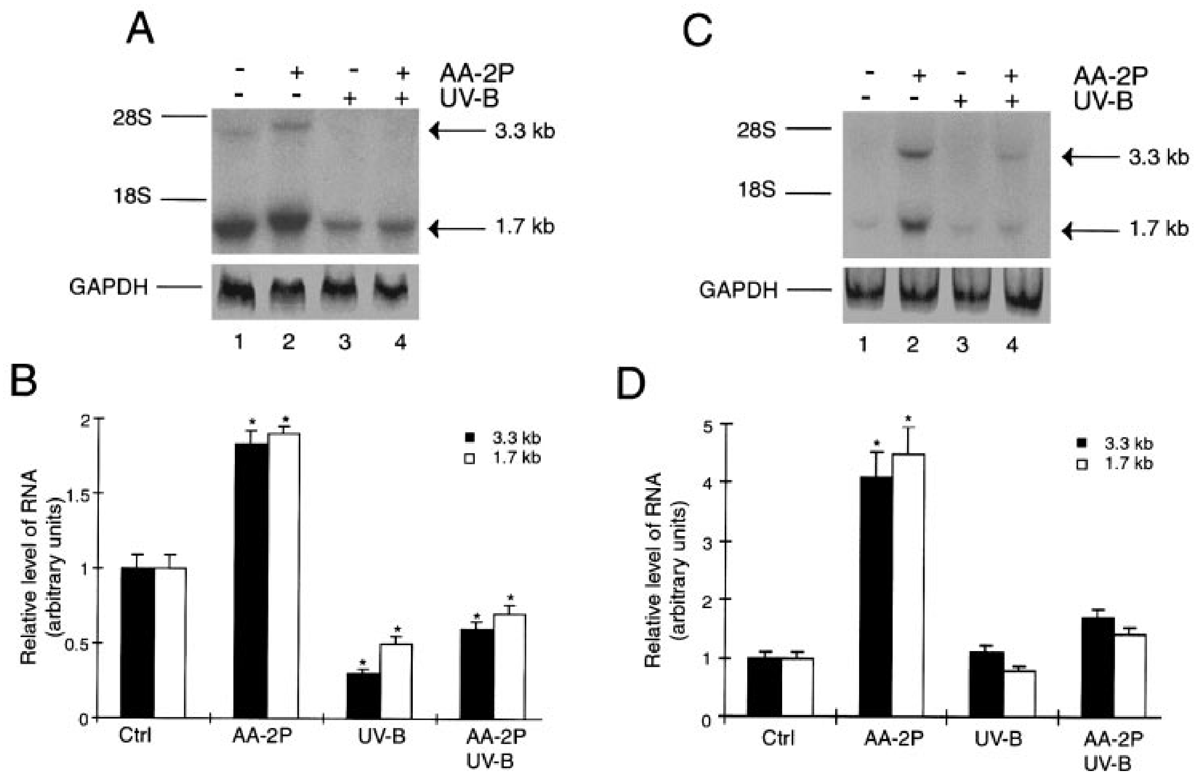

Figure 2 Induction of Fra-1 expression by ascorbate

(**A**) Northern-blot analysis for Fra-1 message in HaCaT cells. Cells were left untreated (lane 1) or irradiated in the absence (lane 3) or presence (lane 4) of 1 mM AA-2P; also shown is the sample incubated with AA-2P alone (lane 2). Positions of the Fra-1 transcripts (3.3 and 1.7 kb) are indicated on the right. GAPDH hybridization, performed as an internal control, is also shown (lower panel). (**B**) Scanning densitometry of autoradiography shown in (**A**). Values are reported as fold increase relative to the control, arbitrarily set to 1, after normalization with GAPDH. Data are the means ± S.E.M. from three separate experiments. Black bars, 3.3 kb transcript for Fra-1; white bars, 1.7 kb transcript for Fra-1. $^*P < 0.05$ versus control by the χ^2 test. (**C**) Northern-blot analysis for Fra-1 message in NHEKs. Details are as for (**A**). (**D**) Scanning densitometry of autoradiography shown in (**C**). Details are as for (**B**). $^*P < 0.001$ versus the control by χ^2 test.

of Biochemistry, German Cancer Research Center, Heidelberg, Germany). Cells were grown in a 1:1 mixture of minimal essential medium and Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 1.2 g/l sodium bicarbonate, 1% (v/v) non-essential amino acids and 15 mM Hepes, at 37 °C with 5% CO_2 in a humidified atmosphere. No antibiotics were used. Cells were split 1:6 twice weekly and fed 24 h before each experiment. NHEKs were obtained from Clonetics (San Diego, CA, U.S.A.) and grown in calf skin collagen-coated dishes in serum-free keratinocyte growth medium (SFM, Gibco) with 0.05 mM calcium, supplemented with 60 μg/ml bovine pituitary extract. Third-passage NHEKs were used for each experiment.

For irradiation, cells were exposed to UV-B (77 mJ/cm^2; 1 min exposure in PBS) followed by incubation for 4 h before harvesting. When indicated, 1 mM AA-2P was added to the cells 5 h prior to UV irradiation. Controls were represented by untreated keratinocytes or cells incubated with AA-2P alone.

cDNA expression array

Differential gene expression was performed by using Atlas™ cDNA Expression Array (Clontech, Palo Alto, CA, U.S.A.), according to the manufacturer's protocol. Briefly, total RNA was purified from HaCaT cells that had been subjected to different treatments by using the RNeasy kit (Qiagen, Hilden, Germany); after incubation with RNase-free DNase I, poly(A)$^+$ RNA was isolated by the use of the oligotex mRNA kit (Qiagen). Poly(A)$^+$ RNA was reverse-transcribed by using the reagents provided and [α-^{32}P]dATP (3000 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.). Array hybridization was carried out overnight with the radioactively labelled cDNA probes; after high-stringency washes, membranes were exposed to X-ray film and analysed by autoradiography.

Northern-blot analysis

Total RNA was purified from NHEKs or HaCaT cells by a guanidinium isothiocyanate method (Trizol; Gibco); 20 μg were electrophoresed through a 1% (w/v) agarose gel in 12 mM Tris/6 mM acetate/0.3 mM EDTA buffer and then blotted on to a nylon membrane (Hybond-N; Amersham). Hybridization was performed in 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, 5 × Denhardt's solution, 50 mM sodium phosphate buffer, 1% (w/v) SDS, 50% (v/v) formamide, 20 μg/ml denatured salmon sperm DNA and 1.5 × 10^6 c.p.m./ml ^{32}P-labelled *fra-1* probe [specific radioactivity, (8–9) × 10^8 c.p.m./μg], at 42 °C for 16–18 h. After high-stringency washes, membranes were analysed by autoradiography.

fra-1 cDNA probe was generated by reverse transcriptase PCR, using 5′- and 3′-specific primers corresponding to bases 124–143 and 434–455 (accession number X16707), and the 332 bp amplification product purified by Concert Rapid PCR Purification System (Gibco). *GAPDH* cDNA probe was also generated by reverse transcriptase PCR, using 5′- and 3′-specific

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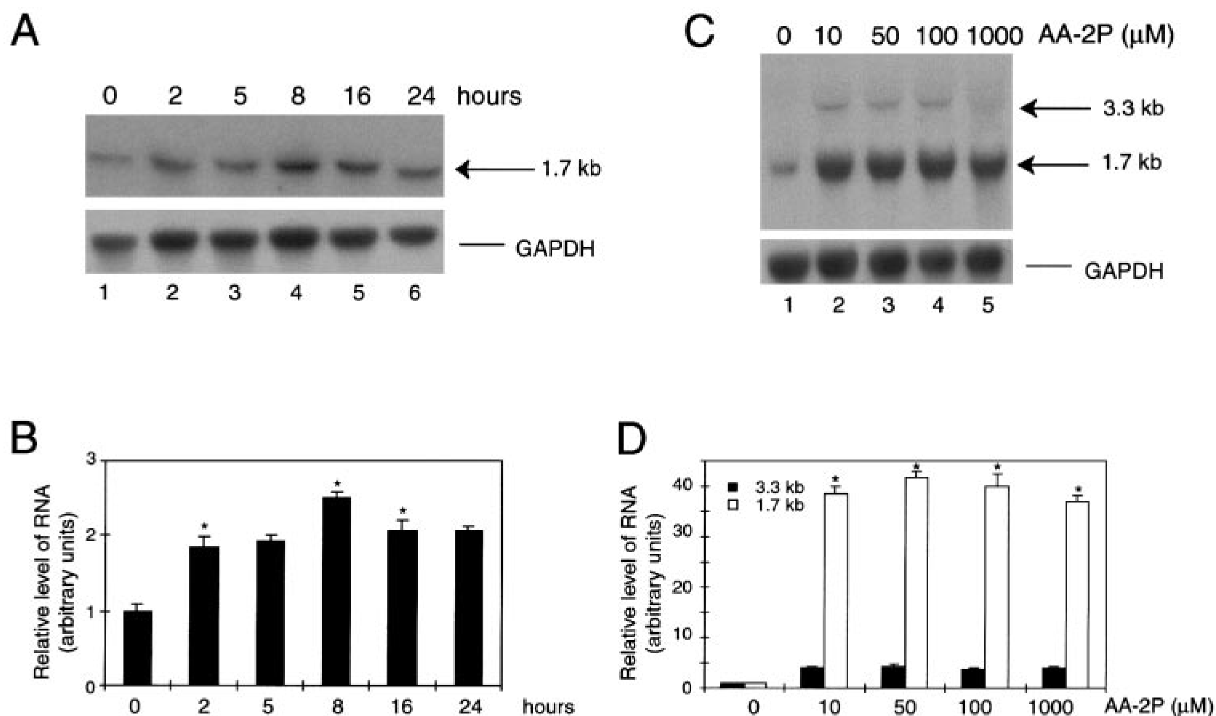

Figure 3 Time course and dose-response experiments of Fra-1 expression

(**A**) Time course of Fra-1 mRNA expression. HaCaT cells were incubated with 1 mM AA-2P for the indicated times (lanes 2–6). Total RNA was then extracted and subjected to Northern-blot analysis. Lane 1, control cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) hybridization is shown in the lower panel. (**B**) Scanning densitometry of autoradiography shown in (**A**). Values are reported as fold increase relative to the control, arbitrarily set to 1, after normalization with GAPDH. Data are the means ± S.E.M. from three different experiments. $^{*}P < 0.05$ versus the control (χ^2 test). (**C**) Dose–response experiments. HaCaT keratinocytes were loaded with the indicated concentrations of AA-2P for 5 h and then Fra-1 expression was assessed by Northern blotting. Lane 1, control cells; lanes 2–5, cells incubated with 10, 50, 100 and 1000 μM AA-2P, respectively. GAPDH hybridization is again shown in the lower panel. (**D**) Scanning densitometry of autoradiography shown in (**C**). Details are as for (**B**). Black bars, 3.3 kb transcript for Fra-1; white bars, 1.7 kb transcript for Fra-1. $^{*}P < 0.001$ versus control (χ^2 test).

primers corresponding to bases 71–92 and 215–234 (accession number M33197), and the 164 bp amplification product purified by Concert Rapid PCR Purification System.

Electrophoretic mobility-shift assay and supershift analysis

Nuclear mini-extracts obtained from 10^7 cells were prepared according to Schreiber et al. [33] with the modifications reported in Lee et al. [34]. Mobility-shift experiments were performed as described in [34].

The AP-1 consensus oligonucleotide was 5′-CGCTTGATG-AGTCAGCCGGAA-3′ and the AP-1 mutant oligonucleotide was 5′-CGCTTGATTAGTTAGCCGGAA-3′. The complexes were resolved on non-denaturing 6 % (w/v) polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer (90 mM Tris/90 mM borate/ 2.5 mM EDTA) for 1 h at 14 V/cm and autoradiographed overnight.

For supershift analysis, mini-extracts were preincubated with 3 μg of specific antiserum for 2 h at 4 °C before addition of ^{33}P-labelled oligonucleotide. Complexes were then processed as described above. Anti-Fra-1 and anti-c-Fos antibodies were from Santa Cruz Biotechnology.

Transient transfections of keratinocytes

Cells were transfected with a 3 × AP-1 pBLCAT3 construct [35]: the reporter vector consists of three wild-type or mutated AP-1 tandem repeats upstream of the chloramphenicol acetyltransferase (*CAT*) structural gene. Transient transfections were performed in triplicate using Effectene reagent (Gibco) according to the manufacturer's protocol and the efficiency of transfection was monitored by using the cytomegalovirus β-galactosidase construct (pCMV-β, Clontech). Briefly, 2×10^5 cells were plated in each 35 mm well of six-well culture plates the day before transfection. For each well, 0.4 μg of reporter plasmid and 0.1 μg of pCMV-β were mixed with 3.2 μl of Enhancer reagent (Gibco) and 10 μl of Effectene reagent and then incubated for 10 min at room temperature. After incubation, the mixture was added to each well and incubated for 16–24 h before replacing the transfection medium. After transfection (24 h), cells were irradiated immediately or after 5 h of loading with 1 mM AA-2P and then incubated for an additional 4 h. After that, cells were harvested and lysed in 0.4 ml of 5 × reporter lysis buffer (Promega, Madison, WI, U.S.A.) and then CAT activity was assayed as described in [36]. CAT activities were normalized by protein content and β-galactosidase activity.

JNK and c-Jun phosphorylation

Cells were lysed in RIPA buffer containing 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM PMSF, 20 μg/ml leupeptin and 20 μg/ml aprotinin. After centrifugation and protein determination, 500 μg of each lysate were incubated on ice with 2 μg of anti-JNK1 (C17) or anti-c-Jun (H79) antibodies for 2 h. Immune complexes were then collected by Protein A–agarose for 25 min

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and washed thrice with RIPA buffer, containing 20 mM β-glycerophosphate, 1 mM sodium orthovanadate and 0.5 mM dithiothreitol. After recovering of the immune complexes, samples were subjected to SDS/PAGE (12.5 % gel, w/v), and then blotted on to a PVDF membrane. Phosphorylated JNK or c-Jun were detected by using anti-phospho-JNK (G7) or anti-phospho-c-Jun (Ser 63) antibodies. The amounts of the immunoprecipitated JNK1, as well as of the immunoprecipitated c-Jun, were analysed on the same filter by Western blotting (anti-JNK1, C17; anti-c-Jun, H79).

RESULTS

Ascorbate influences gene expression in UV-B-treated keratinocytes

We investigated the ability of ascorbate to interfere with gene expression. To this end, HaCaT cells were either irradiated with UV-B or pre-incubated for 5 h with 1 mM AA-2P before irradiation; both samples were then incubated for an additional 4 h before harvesting. Untreated or AA-2P-treated (1 mM, 5 h) keratinocytes were used as non-irradiated controls. In order to identify transcripts which could be differentially regulated in irradiated keratinocytes in the presence or absence of ascorbate, we used cDNA microarray hybridization, as it allows simultaneous screening of 588 mRNAs involved in different biologicalprocesses (e.g. cell cycle, signal transduction, regulation of transcription, cell death, cell–cell communication and stress response; refer to Clontech, manufacturer of the Atlas™ cDNA Expression Array, for specific details).

Figure 1 shows a representative array hybridization. The expression of several genes was modulated by the specific treatments. In comparison with untreated cells (Figure 1A), UV-B modified the expression pattern, including that of transcription factors (e.g. the AP-1 complex) and cell-cycle proteins (compare Figures 1B and 1A). Changes in expression of some of these genes have already been described, such as the down-regulation of c-*myc* (Figures 1A and 1B, square 1). In addition, UV-mediated regulation of novel genes was identified, such as the inverse regulation of ETR101 and ETR103 (EGR1) mRNAs, which belong to the EGR (early growth response) family of transcription factors (Figures 1A and 1B, squares 4 and 5) [37].

AA-2P-treated cells displayed up-regulation of *fra-1*, *GST-pi* and *MLH1* expression (compare Figures 1C and 1A, lines 1–3). This ascorbate-mediated positive modulation of *fra-1*, *GST-pi* and *MLH1* was partially counteracted by UV-B (Figure 1D). This result also indicates that ascorbate does not mask in a non-specific manner the biological effects of UV-B by simply interfering with their absorbance.

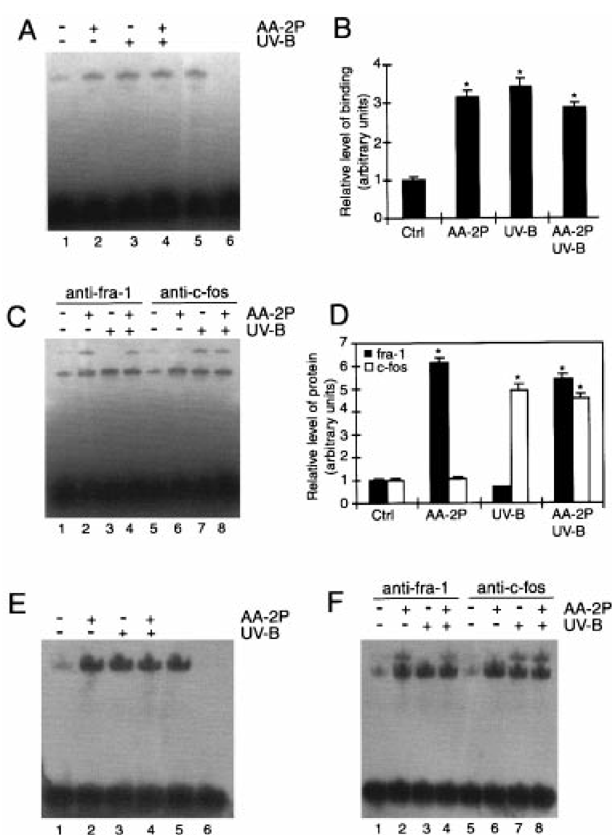

Figure 4 Effect of ascorbate supplementation on AP-1 activity

(**A**) Effect of ascorbate on AP-1 DNA-binding activity. Nuclear extracts were prepared from untreated cells (lane 1) or from keratinocytes incubated with 1 mM AA-2P (lane 2) or irradiated with UV-B in the absence (lane 3) or presence (lane 4) of AA-2P. A molar excess of AP-1 mutant oligonucleotide and AP-1 consensus oligonucleotide (lanes 5 and 6) were used as specificity controls. (**B**) Scanning densitometry of autoradiography shown in (**A**). Values are reported as fold increase relative to the control, arbitrarily set to 1. Data are the means ± S.E.M. from three different experiments. $^{*}P < 0.05$ versus the control (χ^2 test). (**C**) Supershift experiment of AP-1 components. Nuclear extracts were incubated with AP-1-specific labelled oligonucleotide together with antisera raised against Fra-1 (lanes 1–4) or c-Fos (lanes 5–8) proteins. Lanes 1 and 5, control cells; lanes 2 and 6, AA-2P-loaded cells; lanes 3 and 7, irradiated cells; lanes 4 and 8, irradiated cells in the presence of AA-2P. (**D**) Scanning densitometry of autoradiography shown in (**C**). Details are as for (**B**). Black bars, AP-1 oligonucleotide supershifted with anti-Fra-1 antibody; white bars, AP-1 oligonucleotide supershifted with anti-c-Fos antibody. $^{*}P < 0.001$ versus the control (χ^2 test). (**E**) Effect of ascorbate on AP-1 DNA-binding activity in NHEKs. Lanes are as for (**A**). (**F**) Supershift experiment of AP-1 components in NHEKs. Lanes are as for (**C**). All autoradiography is representative of three different experiments.

Ascorbate inhibits UV-B-induced AP-1 activity by modulating AP-1-complex composition

Changes in gene-expression levels observed in the Atlas expression profiling, as well as dose- and time-dependence of expression, have been confirmed by Northern-blot analysis on HaCaT cells collected after each treatment.

AA-2P led to an increase in both RNA transcripts described for Fra-1 [38] in HaCaT cells, which was otherwise influenced by UV-B in a negative fashion (Figures 2A and 2B). In addition, UV irradiation only partially counteracted the positive regulation of ascorbate: indeed, the expression of both messengers was affected by UV-B treatment, but not completely abolished. A similar result was obtained with normal keratinocytes: ascorbate-supplemented NHEKs showed a 4–5-fold induction of *fra-1* expression, which was partially counteracted by UV-B treatment (Figures 2C and 2D).

We also investigated the time- and dose-dependence of ascorbate-mediated modulation of *fra-1* expression. As shown in Figure 3(A), with intracellular saturating levels of ascorbate (1 mM AA-2P exogenously) [30], the positive modulation was evident after just 2 h of incubation. The major transcript of 1.7 kb reached maximal induction at 8 h and steady high levels were seen over a period of 24 h (Figures 3A and 3B). In dose–response experiments, we found that AA-2P concentrations as low as 10 μM were potent inducers of *fra-1* expression, as assessed by Northern blotting (Figure 3C, lane 2). Moreover, we did not find any difference in terms of induction with concen-

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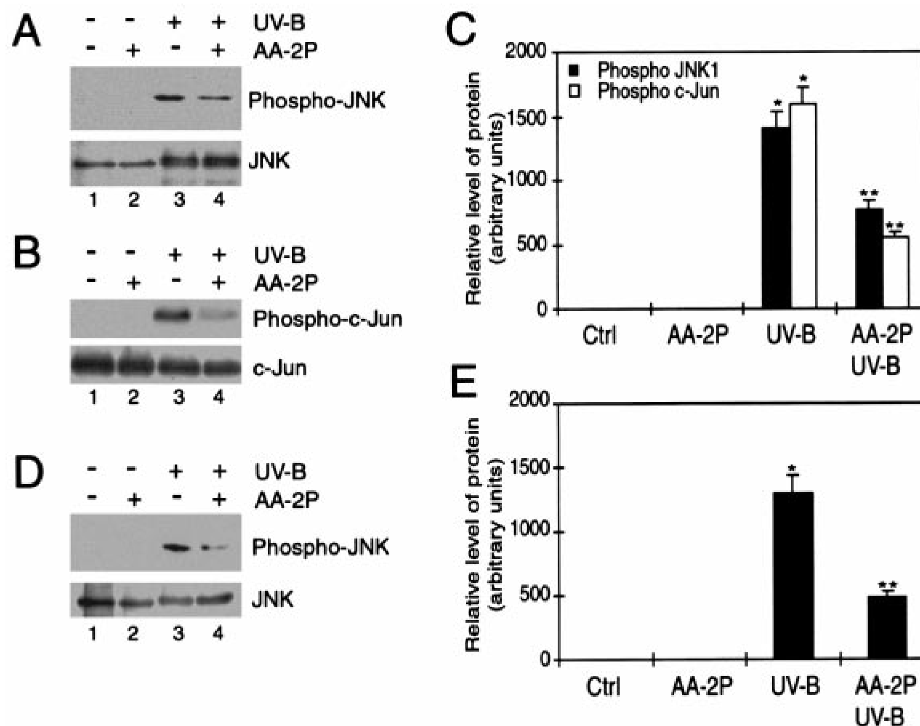

Figure 5 Effect of ascorbate loading on JNK activity

(**A**) Effect of ascorbate supplementation on JNK phosphorylation. HaCaT cells were left untreated (lane 1), incubated with 1 mM AA-2P alone (lane 2) or treated with UV-B in the absence (lane 3) or presence (lane 4) of AA-2P. Phosphorylated JNK was detected by immunoblotting using an anti-phospho-JNK antibody (upper panel). In the lower panel is shown the amount of the immunoprecipitated JNK1 on the same filter after re-probing with antibodies to JNK. (**B**) c-Jun phosphorylation after ascorbate supplementation. Phosphorylation levels of endogenous c-Jun protein were analysed after each treatment by anti-phospho c-Jun antibody (upper panel). The amount of the immunoprecipitated c-Jun was detected on the same filter with an anti-c-Jun antibody (lower panel). Lanes are as for (**A**). (**C**) Scanning densitometry of autoradiography shown in (**A**) and (**B**). Values are reported as fold increase relative to the control, arbitrarily set to 1, after normalization with the levels of immunoprecipitated proteins. Results are the means ± S.E.M. from three different experiments. Black bars, phospho-JNK1; white bars, phospho-c-Jun. *$P < 0.001$ versus control; **$P < 0.001$ versus UV-B (χ^2 test). (**D**) Effect of ascorbate supplementation on JNK phosphorylation in normal keratinocytes. Lanes are as for (**A**). Phosphorylated JNK was detected by immunoblotting (upper panel). In the lower panel is shown the amount of the immunoprecipitated JNK1 on the same filter after re-probing with antibodies to JNK. (**E**) Scanning densitometry of autoradiography shown in (**C**). Details are as for (**C**). *$P < 0.001$ versus control; **$P < 0.001$ versus UV-B (χ^2 test).

trations of AA-2P ranging from 10 μM to 1 mM, as indicated by densitometry analysis (Figure 3D). This suggests that the up-regulation of Fra-1 mRNA may be saturable with low concentrations of ascorbate. Indeed, experiments carried out with concentrations lower than 10 μM showed that 1 μM AA-2P did not change the amounts of Fra-1 mRNA, but with 5 μM AA-2P a positive response was seen, although a maximum effect could be reached only with 10 μM AA-2P (results not shown).

To elucidate the effects of ascorbate-induced *fra-1* expression, we investigated the AP-1 DNA-binding activity as well as the composition of AP-1 complexes after each treatment in HaCaT cells. As shown in Figures 4(A) and 4(B), all treatments increased the ability of AP-1 complexes to bind an oligonucleotide containing the specific AP-1 consensus sequence (Figure 4A, lanes 2–4). Specificity of the binding was confirmed by the use of AP-1 mutated sequence and a molar excess of unlabelled AP-1 consensus oligonucleotide (Figure 4A, lanes 5 and 6). Incubation with IgG, used as an internal control, had no effect (results not shown). We then analysed by supershift experiments the composition of AP-1 complexes: we used antisera raised against Fra-1 and c-Fos in order to determine whether different treatments led to significant changes in the formation of AP-1 complexes. We found that, in HaCaT keratinocytes treated with 1 mM AA-2P (Figure 4C, lanes 2 and 6), Fra-1 was significantly increased (6.2 ± 0.6-fold induction over untreated cells; see Figure 4D), whereas c-Fos was comparable with control cells. In contrast, in irradiated cells, AP-1 dimers contained c-Fos protein (4.9 ± 0.3-fold induction over untreated cells; see Figure 4D), whereas Fra-1 was almost absent (Figure 4C, lanes 3 and 7). Finally, irradiated cells in the presence of ascorbate exhibited a different pattern, in which AP-1 complexes contained both proteins. The same pattern of expression was also seen in normal keratinocytes (see Figures 4E and 4F). Thus ascorbate was able to change the nature of the AP-1 complex in irradiated cells by increasing the steady-state levels of Fra-1 protein.

Ascorbate reduces the activation of JNK1 in response to UV-B radiation

As the AP-1 transcription factor is activated through the JNK signalling pathway, we tested whether vitamin C also exerted its negative regulatory functions by acting upstream, by specifically modulating JNK activity.

We performed Western-blot analysis with anti-JNK or anti-phospho-JNK antibodies in order to establish whether vitamin C affected the level of JNK and/or the degree of JNK phosphorylation. Phosphorylated JNK was detected after treatment with UV-B irradiation (Figure 5A, upper panel, lane 3), whereas, in ascorbate-loaded cells, the UV-B-induced JNK phosphorylation was decreased (Figure 5A, upper panel, lane 4).

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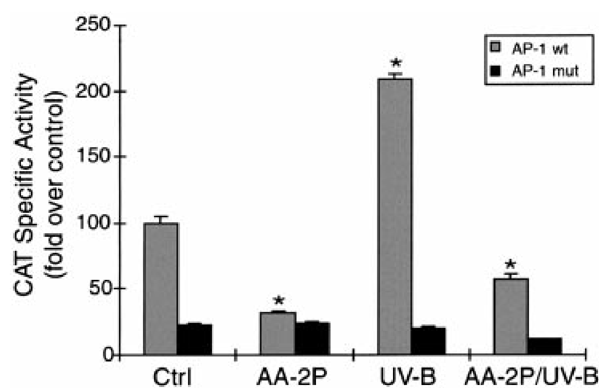

Figure 6 Effect of ascorbate on AP-1-driven transcription

HaCaT cells transiently transfected with a wild-type (grey columns) or a mutated (black columns) AP-1-driven CAT construct were left untreated or irradiated with UV-B. Where indicated, cells were also pre-incubated with 1 mM AA-2P for 5 h. Data are expressed as the percentage of CAT activity relative to untreated cells, arbitrarily set to 100%. Results are means $\pm$ S.E.M. from triplicate determinations carried out in three different experiments. $^{*}P < 0.001$ versus the control (χ^2 test).

As revealed by densitometry analysis (Figure 5C), vitamin C led to a 50 % inhibition of phosphorylation. In any case, the level of JNK protein was not affected by ascorbate treatment (Figure 5A, lower panel). A marked inhibition of JNK1 phosphorylation was also observed in NHEKs treated with ascorbate (Figures 5D and 5E).

Next, we studied whether the inhibition of JNK phosphorylation resulted in the inhibition of kinase activity. To this end, we investigated the endogenous c-Jun phosphorylation levels in cellular extracts derived from untreated cells as well as from keratinocytes exposed to UV-B, in the absence or presence of ascorbate. We found that, in both HaCaT cells and NHEKs, UV-B activated JNK, thus increasing phosphorylation of the natural c-Jun substrate (Figure 5B, lane 3, and results not shown), and that ascorbate induced a significant decrease in the amounts of the phosphorylated substrate (Figure 5B, lane 4, and results not shown); in particular, the rate of inhibition for HaCaT cells was 68.7 $\pm$ 3.4 %, as revealed by densitometry analysis (Figure 5C). As the levels of endogenous phosphorylated c-Jun in the AP-1 complex determine transcriptional regulation, the reduction in phospho-c-Jun seen in Figure 5(B) indicates that ascorbate can also act upstream of the AP-1 complex, through the post-translational control of JNK.

Ascorbate inhibits gene transcription via AP-1 motifs

The above experiments indicate that ascorbate can affect the JNK/AP-1 pathway on two distinct levels. We then examined the effect of ascorbate on AP-1-dependent gene transactivation. Keratinocytes were transiently transfected with a reporter vector containing three AP-1 sites upstream of the *CAT* gene and, 24 h after transfection, they were treated with AA-2P alone or in combination with UV-B irradiation. As expected, UV-B treatment led to an increase in AP-1-driven *CAT* transcription of about 2-fold over the control (arbitrarily set to 100 %). On the other hand, vitamin C supplementation was able to inhibit both basal and UV-B-induced CAT activities (Figure 6); mutation of the AP-1 motif completely abrogated transcription of the *CAT* gene.

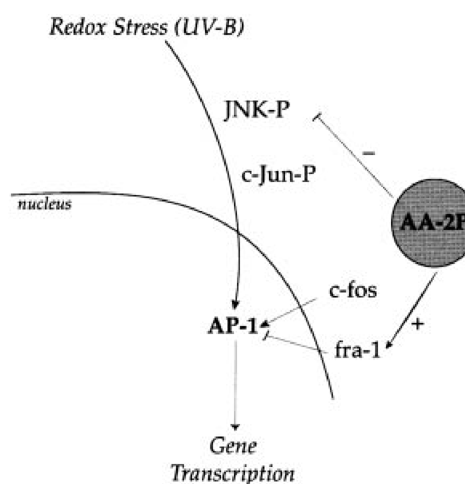

Figure 7 Schematic model of the JNK signalling cascade and ascorbate interference with the pathway

Vitamin C has transcriptional (through the induction of Fra-1 expression) and post-transcriptional (through the inhibition of JNK phosphorylation) control. As a result, the nature of AP-1 complexes were modified and acquired an inhibitory activity; moreover JNK activity, and thus c-Jun phosphorylation, was inhibited. UV-B irradiation exerts opposite effects on the JNK cascade, inducing JNK activity and inhibiting Fra-1 expression. Nonetheless, UV-B irradiation was not able to overcome AA-2P-mediated inhibition.

DISCUSSION

We have demonstrated previously [30] that exogenously provided AA-2P was taken up against a concentration gradient and that it was concentrated inside the cell as the dephosphorylated form by 40-fold compared with extracellular concentrations. Intracellular accumulation of vitamin C made cells more resistant to UV-B cytotoxicity and the protective effect was due mainly to the ability of ascorbate to act as a free-radical trapper [30,39]. In this article we describe an additional mechanism through which vitamin C may prevent UV-induced cell damage: it can act as a modulator of crucial genes.

In order to identify genes whose expression was regulated, we employed cDNA microarray hybridization. Parallel analysis of hybridization signals enabled us to obtain a gene-expression profile in response to UV-B irradiation: most of the identified modulated genes have already been described. However, this is the first report of the overall changes in mRNAs involved in UV-B-induced cell damage. Furthermore, we found at least three transcripts (Fra-1, GST-pi and MLH1) that were up-regulated by ascorbate in spite of subsequent UV-B treatment.

Fra-1 belongs to the Fos superfamily (including c-Fos, Fos B and Fra-2), which heterodimerizes with Jun family members (c-Jun, Jun B and Jun D) to form stable AP-1 transcription-factor complexes [40]. Lacking the C-terminal transactivation domain, Fra-1 is a negative regulator of AP-1 activity; indeed, it antagonizes the transcriptional effects induced by either PMA or expression of c-Jun and c-Fos [41]. All genes involved in formation of the AP-1 complex are clearly redox-regulated; generation of reactive oxygen species upon UV irradiation has been shown to alter the redox state of the cell, thus controlling AP-1-mediated gene expression. Indeed, exposure of keratinocytes to UV-B led to induction of c-*fos*, c-*jun* and *jun B* mRNA levels *in vitro* and *in vivo*; conversely, negative regulators of the complex, such as *fra-1* and *fra-2*, were down-modulated by UV-

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B [42]. Antioxidants, acting on upstream or downstream specific targets, can interfere with this redox-sensitive transcription factor [43]; moreover, enzymic as well as non-enzymic antioxidants have been shown to either activate [44] or inhibit [41,45] AP-1 at the transcriptional or post-transcriptional level. Here, we have demonstrated that ascorbate increased the levels of Fra-1 mRNA and that this induction was not overcome by UV-B radiation. As a result, vitamin C might modulate the cellular response to UV radiation by modifying the nature of AP-1 complexes; in the presence of Fra-1 protein, AP-1 acquires an inhibitory activity. The mechanism underlying the ascorbate-mediated regulation of AP-1 is enhanced further by the inhibition of JNK activity. JNK belongs to a family of stress kinases, transiently activated by intracellular changes in the redox potential elicited by different stressors, such as UV light and H_2O_2. Thus active JNK, phosphorylating several transcription factors, mediates multiple pathways. Among the cellular targets of JNK activity, phosphorylation of c-Jun is of particular interest as it contributes to the transactivation of the above-mentioned AP-1 complex. In our models, vitamin C negatively modulated the JNK activity and, as a consequence, phosphorylation of endogenous c-Jun protein was inhibited. Subsequent exposure of keratinocytes to UV-B was not able to overcome ascorbate-mediated down-regulation, thus maintaining the JNK/AP-1 signalling pathway in an inhibited state (Figure 7).

In conclusion, data presented here provide new insights into the possible mechanisms through which vitamin C mediates the cellular response against UV-induced cell damage. Since AP-1 plays a key role in keratinocyte cell death [46], as well as in cell death induced by DNA damage [47], our findings suggest that its regulation by ascorbate might act in parallel with the antioxidant activity of vitamin C in the prevention of cell death.

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