Characterization of Keratinocyte Differentiation Induced by Ascorbic Acid: Protein Kinase C Involvement and Vitamin C Homeostasis[1]

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Epidermal keratinocytes undergo differentiation in response to several stimuli to form the cornified envelope, a structure that contributes to the barrier function of skin. Although differentiation has been extensively analyzed, the precise role of vitamin C during this process is still not defined. Ascorbic acid, besides acting as a radical scavenger, has been shown to promote mesenchymal differentiation. In this study, we found that keratinocytes grown in ascorbate-supplemented medium developed a differentiated phenotype, as demonstrated by enhanced expression of marker genes and increase in cornified envelope content. The pro-differentiating effects of ascorbate were mediated by the protein-kinase-C-dependent induction of activating protein 1 DNA binding activity; indeed, down-modulation of protein kinase C activity abolished differentiation triggered by ascorbic acid. Although vitamin C appeared to regulate the same signaling pathway modulated by calcium, a classical *in vitro* inducer of epidermal differentiation, nonetheless terminally differentiated keratinocytes exhibited different ascorbate homeostasis and cellular antioxidant status. Indeed, we found that, unlike calcium, differentiation promoted by ascorbate was accompanied by (i) an enhanced ascorbate transport, due to overexpression of specific transporters, (ii) a great efficiency of dehydroascorbate uptake, and (iii) an increase in glutathione content with respect to proliferating cells. Ascorbic acid may be useful to promote epidermal differentiation, avoiding depletion of hydrophilic antioxidant stores. ***Key words: activating protein 1 (AP-1)/dehydroascorbate/glutathione/hSVCT1/hSVCT2. J Invest Dermatol 118:372–379, 2002***

Several studies have identified a role for vitamin C in skin repair, and this molecule is often used *in vivo* for its healing, antiageing, and anticarcinogenic properties. Studies performed with epidermis *in toto* demonstrated that the wound healing process is accompanied by oxidation of ascorbic acid (AA), and that other factors besides collagen synthesis may enhance AA oxidation in the early stages of tissue regeneration (Kim *et al*, 1994); in addition, AA has been shown to be necessary for multistep production of bioengineered skin substitutes (Auger *et al*, 2000).

Epidermal differentiation plays a central role during wound repair. As keratinocytes migrate towards the upper layers of the epidermis, the expression pattern of epidermal-specific genes undergoes drastic changes (Reichert *et al*, 1993). In differentiating keratinocytes, several genes, such as basal keratins K5 and K14, are downregulated, whereas the expression of suprabasal markers, such as K1 and K10, filaggrin, involucrin, loricrin, and transglutaminases (TGases) increases (Eckert *et al*, 1997). All of these proteins are required for correct assembly of the cornified cell envelope (CE), a specialized structure that represents the physical barrier between the body and the environment and protects mammals from mechanical and chemical insults (Hohl, 1990; Nemes and Steinert, 1999). Among the transcription factors regulating tissue- and differentiation-specific expression, the activating protein 1 (AP-1) plays a major role (Welter and Eckert, 1995; Rossi *et al*, 1998). This nuclear transcription factor consists of homodimers or heterodimers between Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD), and activating transcription factor (ATF2, ATF3, B-ATF) family members (Karin *et al*, 1997), which modulate transcription by binding to specific DNA regulatory elements in the promoter region of targeted genes (the TRE and CRE sites) (Hai and Curran, 1991). In the epidermis, the selectivity of genes regulated by AP-1 is determined by distinct AP-1 complexes, which have a differentiation-specific distribution, and by interactions with other transcriptional regulators and/or distal regulatory elements. Thus, the fine-tuned modulation of AP-1 DNA binding activity allows the expression of various differentiation marker genes that have AP-1 responsive elements in their regulatory regions (Lu *et al*, 1994; Yamada *et al*, 1994).

Vitamin C has been proved to regulate cell growth and differentiation in culture models other than keratinocytes.

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Abbreviations: AA, ascorbic acid; AA-2P, L-ascorbate 2-phosphate; AFR, ascorbyl free radical; AP-1, activating protein 1; CE, cornified cell envelope; DHA, dehydroascorbate; DOG, deoxy-D-glucose; GSH, reduced glutathione; TGase, transglutaminase.

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Ascorbate is required for *in vitro* differentiation of several mesenchyme-derived cell types (Farquharson *et al*, 1998; Otsuka *et al*, 1999) and is needed for *in vivo* differentiation of connective tissues, such as bone, muscle, and cartilage (Franceschi, 1992). In particular, the commitment to an osteoblast phenotype requires the formation of a collagen-containing matrix in response to AA, thus providing a permissive environment for the expression of tissue-specific genes, such as osteocalcin and alkaline phosphatase (Xiao *et al*, 1997).

We have recently demonstrated that HaCaT keratinocytes possess very efficient systems for maintaining high levels of intracellular AA, which is accumulated in millimolar concentrations. Human keratinocytes transport both reduced and oxidized forms of vitamin C inside cells through two different mechanisms, an Na^+-dependent cotransporter for AA and a facilitative glucose transporter for dehydroascorbic acid (DHA) (Savini *et al*, 2000). In addition, HaCaT keratinocytes are able to enzymatically recycle vitamin C. DHA is reduced to AA by cytosolic reductases (GSH-NADPH-dependent, lipoic acid-NADH-dependent, and thioredoxin reductase), whereas ascorbyl free radical (AFR) is reduced to AA by plasma membrane, mitochondrial, and microsomal AFR reductases (Savini *et al*, 1999). Moreover, we have shown that accumulated vitamin C protects HaCaT cells against UV-induced cell damage by acting as a scavenger of reactive oxygen species and by interfering at multiple levels with the AP-1 pathway. In normal human epidermal keratinocytes and HaCaT cells, ascorbate increases *fra-1* mRNA levels and negatively modulates JNK activity, thus inhibiting phosphorylation of endogenous c-Jun protein. These effects of AA on the JNK/AP-1 pathway lead to a specific modification of the nature of AP-1 complexes (Catani *et al*, 2001).

On the basis of these findings, we investigated the potential regulatory role of ascorbate in epidermal differentiation. We found that normal human epidermal keratinocytes and HaCaT cells grown in ascorbate-supplemented medium showed a differentiated phenotype, characterized by the expression of different marker genes and CE formation. The pro-differentiating effects of AA were mediated by induction of AP-1 activity, together with induction of its upstream regulator, protein kinase C (PKC), a kinase known to be essential for regulation of AP-1 (Rutberg *et al*, 1996). Moreover, we found that ascorbate-triggered differentiation was accompanied by a more favorable antioxidant status with respect to keratinocytes differentiated by calcium, the classical inducer of epidermal differentiation. Indeed, AA-induced differentiation increased glutathione levels and had specific effects on vitamin C metabolism, including AA transport and recycling.

MATERIALS AND METHODS

Reagents Magnesium ascorbic acid-2 phosphate (AA-2P) was obtained from Wako Pure Chemical Industries (Neuss, Germany). [α-^{33}P]-dCTP, [γ-^{33}P]-ATP, and [^{14}C]-AA were purchased from Amersham (Arlington Heights, IL). DHA was obtained from ICN (Aurora, OH). Anti-K1 antibody was from Babco (Berkeley, CA). Bisindolylmaleimide GF 109203X (PKC inhibitor) was from Calbiochem (La Jolla, CA). All the other reagents, unless otherwise indicated, were from Sigma Chemical (St. Louis, MO).

Cell cultures Cryopreserved normal human epidermal keratinocytes (newborn foreskin) were obtained from Clonetics (San Diego, CA) and grown in dishes coated with calf skin collagen type III (100 mg per ml in 0.1 M acetic acid) in serum-free keratinocyte growth medium (Gibco, Berlin, Germany) at 0.05 mM calcium, supplemented with 60 μg per ml of bovine pituitary extract. Third passage normal human epidermal keratinocytes were used for each experiment. HaCaT cells (Boukamp *et al*, 1988) were kindly provided by Prof. N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany, and were grown in a 1:1 mixture of minimal essential medium and Ham's F-12 medium (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (HyClone, Oud-Beijerland, Holland), 1.2 g per l Na bicarbonate, 1% (vol/vol) nonessential 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.

Determination of CEs CEs were extracted from proliferating or differentiating cells by exhaustive boiling and sonication in 2% sodium dodecyl sulfate (SDS), 20 mM dithiothreitol (DTT), 0.1 M Tris–HCl pH 8.0, and 0.5 mM ethylenediamine tetraacetic acid (EDTA), as described previously (Steven and Steinert, 1994). CEs were quantified by spectrophotometry at 600 nm.

Reverse transcription polymerase chain reaction (RT-PCR) Two to five million normal human epidermal keratinocytes and HaCaT cells were used to isolate total RNA by Trizol (Gibco). Amplification of TGase 1, loricrin, and 18S rRNA was performed as described previously (Rossi *et al*, 2000).

For amplification of sodium-dependent ascorbate transporters (hSVCT1 and hSVCT2), 1 μg of total RNA was reverse transcribed using the Superscript Preamplification System and oligo-dT primer (Gibco), following the manufacturer's instructions; 10% of the first strand reaction was then PCR amplified. Control reactions were performed in order to ensure complete removal of DNA. PCR amplification conditions were carefully examined to stop the reaction during the exponential phase of amplification. The PCR was carried out in the presence of 3 μCi of [α-^{33}P]-dCTP. The amplification parameters were as follows: 94°C, 30 s; 60°C, 30 s; 68°C, 2 min. Linear amplification was observed after 20 cycles. Twenty microliters of the reaction were electrophoresed on a 6% (wt/vol) polyacrylamide gel, which was then dried and autoradiographed. The primers were as follows: hSVCT1 (+) 5′-473 ATTTGGCACCCACGGATACG 492-3′; (–) 5′-851 TCAAGGTCAGGACATAGCAGAGC 829-3′ (Genbank accession number AF170911); hSVCT2 (+) 5′-57 AGAAGGCAAATACGAAGACGAGG 79-3′; (–) 5′-601 GCTCTGCTGTTCCATTGGCAAC 580-3′ (Genbank accession number AJ269478).

Products were validated by size determination and sequencing.

Western blot After each treatment, cells were resuspended in lysis buffer (25 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.5% SDS) and sonicated. 10–20 μg of total proteins were subjected to SDS polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then electroblotted onto a PVDF membrane. Blots were blocked with 5% nonfat dry milk (Biorad, Hercules, CA) and then incubated with anti-K1 primary antibody (Babco). After washings and incubation with the horseradish-peroxidase-conjugated secondary antibody, detection was carried out with enhanced chemiluminescence (Amersham).

Electrophoretic mobility shift assay (EMSA) Nuclear extracts were prepared as previously described (Schreiber *et al*, 1989; Lee *et al*, 1996). The oligonucleotides were AP-1 consensus CGCTTGATGAGTCAGCCGGAA and AP-1 mutant CGCTTGATTAGTTAGCCGGAA. The oligonucleotides were end-labeled with [γ-^{33}P]-ATP and mobility shift experiments were performed as described previously (Lee *et al*, 1996). The complexes were resolved on nondenaturing 6% (wt/vol) polyacrylamide gels in 0.5 × TBE buffer for 1 h at 14 V per cm and autoradiographed overnight.

Transient transfections of keratinocytes Transient transfections were performed in triplicate using lipofectin (Gibco) following the manufacturer's instructions. Cells were transfected as described previously (Rossi *et al*, 1998) with both wild-type and AP-1 mutated minimal loricrin promoters placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (Rossi *et al*, 1998); the transfection efficiency was monitored by using a thymidine kinase β-galactosidase (β-gal) construct (Clontech, Palo Alto, CA). After transfection, cells were treated with 1 mM AA-2P for 5 h before harvesting. CAT activity was assayed using the CAT Enzyme Assay System (Promega, Madison, WI), according to the manufacturer's protocol. β-gal activity was performed using the β-galactosidase Enzyme System (Promega), again according to the manufacturer's protocol. CAT activities were normalized by protein content and β-gal activity. The relative CAT values are the average of three independent experiments, each performed in triplicate.

PKC activity After each treatment, cells were washed twice with ice-cold PBS, resupended in homogenization buffer A [20 mM Tris–HCl pH 8.0, 10 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 2 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg per ml aprotinin, 10 μg per ml leupeptin], and sonicated. After centrifugation at 100,000 × *g* for 1 h at 4°C, the supernatant was collected as the cytosol fraction and the pellet, resuspended in homogenization buffer B (1% Triton X-100 in buffer A), was sonicated for 10 s. The suspension was then centrifuged at 15,000 × *g* for 15 min at 4°C and the supernatant was collected as the membrane fraction. Two micrograms of proteins from membrane and cytosol fractions were assayed for PKC activity in reaction buffer [20 mM Tris–HCl, pH 7.5,

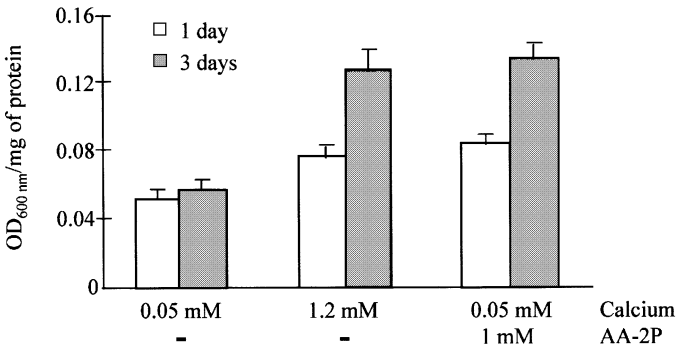

Figure 1. CE formation is induced by ascorbate. Normal human keratinocytes were grown for 1 d (*white bars*) or 3 d (*gray bars*) either in 0.05 or 1.2 mM $CaCl_2$ or in 1 mM AA-2P. CEs were extracted by exhaustive boiling and sonication and then quantified by spectrophotometry at 600 nm. Results are shown as OD600 per mg of protein. Data are the means ± SD of three independent experiments.

5 mM Mg acetate, 10 μg per ml phosphatidylserine, with or without 1 μg per ml dioctanoyl-glycerol vesicles, 0.1 mM $CaCl_2$, 25 μM acylated myelin basic protein substrate (fragment 4–14), 0.01 mM ATP] containing 5 μCi [γ-^{33}P]-ATP. Reactions were incubated for 10 min at 30°C, spotted on P81 paper (Whatmann), and washed four times in 75 mM H_3PO_4. The bound radioactivity was determined with an LKB 1217 RACKBETA liquid scintillation spectrometer. To calculate the amount of radioactivity specifically incorporated into the peptide substrate, the nonspecific binding to P81 paper was determined performing the reaction in the absence of peptide. Experiments were also carried out with the same mixture as above plus 50 μM PKC pseudosubstrate (RFARKGALRQKNVHEVKN) (House and Kemp, 1987) to ensure the specificity of reaction.

AA and DHA uptake Uptake assays were carried out by using both high performance liquid chromatography (HPLC) with ultraviolet detection and scintillation spectrometry. HPLC analysis was performed as described previously (Savini *et al*, 2000). Briefly, 2.5×10^6 cells were incubated in transport medium (5 mM KCl, 1.9 mM KH_2PO_4, 5.5 mM glucose, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM HEPES, 147 mM NaCl, 1.1 mM Na_2HPO_4 pH 7.4) containing AA (with 1 mM DTT to prevent AA oxidation) or DHA. After incubation at 37°C, AA was directly extracted with ice-cold 70% methanol containing 1 mM EDTA and the remaining cell monolayer was analyzed for protein content by the method of Bradford (1976). As DHA does not adsorb at 265 nm (absorbance peak of AA), it was quantified by treating the samples with 10 mM DTT for 10 min.

For scintillation spectrometry measurements, 8×10^5 cells in a 9 cm^2 dish were incubated in 300 μl of transport medium containing [^{14}C]-AA (specific activity 7 mCi per mmol) or [^{14}C]-DHA. The latter compound was prepared by incubating [^{14}C]-AA with 2 U of ascorbate oxidase (Boehringer Mannheim, Mannheim, Germany) immediately before it was added to the incubation medium; complete oxidation was verified by HPLC. In time-dependent experiments, 0.2 μCi of the labeled compound was used. For deoxyglucose (DOG) uptake, the incubation medium contained 0.2 μCi of [1,2-^{3}H]-2-DOG (50 Ci per mmol) and 0.3 mM of 2-DOG. At fixed intervals, cells were harvested, lyzed, and assayed by liquid scintillation spectrometry as described previously (Savini *et al*, 2000). For accumulation studies, cells were incubated with 0.1–0.4 μCi [^{14}C]-AA or [^{14}C]-DHA or [1,2-^{3}H]-2-DOG and adequate concentrations (0–10 mM of AA, 0–15 mM of DHA, and 0–3 mM of DOG, respectively) of the respective unlabeled compounds.

Glutathione content Intracellular reduced and oxidized glutathione content was quantified by a DTNB-glutathione reductase recycling assay, according to the method of Anderson (1985).

Enzymatic activities DHA reductase activity was measured as previously described (Savini *et al*, 2000). Samples (containing 0.2–0.3 mg of protein) were incubated at 37°C for 20 min in 50 mM Tris–HCl pH 7.5 containing 1 mM EDTA and 1 mM DHA. After incubation and precipitation of proteins by methanol, ascorbate content was evaluated by HPLC. To test NADPH/GSH- and NADH/lipoic-acid-dependent DHA reductase activities, single cofactors were added to the reaction mixture. Corrections have been made for nonenzymatic reduction of DHA by endogenous reduced glutathione (GSH).

AFR-reductase activity was measured spectrophotometrically, through the rate of ascorbate-free-radical-dependent oxidation of NADH, by monitoring the decrease in 340 nm absorbance (E = 6.2 per mM per cm) at 25°C (Savini *et al*, 1999). Corrections have been made for direct oxidation of NADH by homogenates. The assay mixture contained 0.05 mM Tris–HCl buffer pH 7.8, 1 mM EDTA, 0.1 mM NADH, 1 mM ascorbate, and aliquots of each sample. The reaction was started by adding 0.28 U of ascorbate oxidase to generate ascorbate free radical.

Transmembrane AFR-reductase activity was measured as previously described, by monitoring at 265 nm the prevention of ascorbate autoxidation in the presence or in the absence of cells (Savini *et al*, 1998).

Thioredoxin reductase activity was assessed by an insulin-reducing assay (Holmgren and Bjornstedt, 1995).

Statistics and kinetic calculations Statistical analysis of means ± SD was conducted with the program Stat View 4.02 for Macintosh (Abacus Concept, Berkeley, CA).

Kinetics for all substrates were determined when transport was linear. The apparent K_m values were obtained by fitting the initial uptake velocity *versus* substrate concentration data directly to the Michaelis–Menten equation. The apparent K_m values were obtained using a Sigma Plot vs 101 program (Jandel Corporation).

RESULTS

Ascorbate promotes CE formation *in vitro* To investigate the possibility that human epidermal keratinocytes undergo differentiation after loading with ascorbate, we studied whether vitamin C could affect the process of CE formation. We employed AA-2P, as it is a stable vitamin C derivative that does not generate oxidation and degradation products in the culture medium and is taken up as ascorbate after cleavage by plasma membrane phosphatases. Ascorbate is then accumulated against a concentration gradient with a specific transport system saturable at about 1 mM extracellular concentration (Savini *et al*, 1999).

Proliferating keratinocytes can be induced to differentiate *in vitro* by changing Ca^{2+} concentrations in the culture medium; indeed, epidermal keratinocytes, grown in high calcium medium, express differentiation-specific genes and form insoluble CEs (Hennings *et al*, 1980). Therefore, we extracted and quantified CEs obtained from proliferating or differentiating normal human epidermal keratinocytes and compared them with CEs from ascorbate-supplemented cells. To this end, normal human epidermal keratinocytes grown in low calcium medium (0.05 mM $CaCl_2$) were shifted to 1.2 mM $CaCl_2$ (high calcium), or treated with 1 mM AA-2P, and cultured for an additional 1 and 3 d before harvesting; the formation of CEs was then monitored by spectrophotometric analysis as a marker of terminal differentiation.

As shown in **Fig 1**, human keratinocytes significantly increased their CE content just 24 h after raising calcium concentrations (*white bars*); the percentage of CEs further increased (about 2.5-fold over control cells) after 72 h (*gray bars*). We found that AA-2P-treated human keratinocytes showed the same CE content as cells cultured in 1.2 mM $CaCl_2$, thus suggesting a differentiating role for vitamin C in epidermis.

Similar results were obtained with the spontaneously immortalized HaCaT cells induced to differentiate by growing them over confluence in the presence of 1.5 mM $CaCl_2$ or in AA-2P-supplemented medium, although these cells were less efficient in forming CEs (data not shown).

Ascorbate-mediated keratinocyte differentiation is linked to induction of PKC activity Calcium-dependent differentiation requires activation of the PKC signaling cascade, which is essential for the upregulation of AP-1-dependent gene expression. In order to gain further insight into the possible mechanism accounting for the differentiating properties of ascorbate, we assayed PKC activity in primary normal human epidermal keratinocytes grown either in high calcium conditions or in ascorbate-supplemented medium.

As shown in **Fig 2**, the total level of PKC activity was increased in both Ca^{2+}-treated (*white bars*) and AA-2P-treated (*gray bars*)

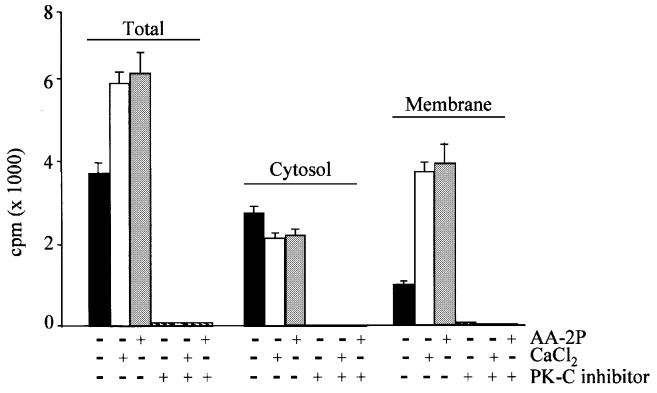

Figure 2. Ascorbate-mediated differentiation involves PKC activation. PKC activity of normal human keratinocyte cells. Keratinocytes were grown either in high calcium conditions (*white bars*) or in AA-2P-supplemented medium (*gray bars*); after incubation, proteins from membrane and cytosol fractions were assayed for PKC activity as described in *Materials and Methods*. Total PKC activity was also measured and compared to proliferating cells grown in low calcium medium (*black bars*). Experiments were also carried out with 50 μM PKC pseudosubstrate (RFARKGALRQKNVHEVKN) to ensure specificity of the reaction (*hatched bars*). Data are the means ± SD of triplicate determinations carried out in three different experiments.

normal human epidermal keratinocytes. Moreover, subcellular fractionation revealed that, in untreated cells (*black bars*), more than 70% of total PKC activity was recovered in the soluble, cytosolic fraction, thus suggesting that the enzyme was in an inactive state; in contrast, in keratinocytes incubated with high Ca^{2+} or with AA-2P, PKC underwent a redistribution to the particulate fraction, suggesting that the enzyme was activated through membrane association. Induction of kinase activity was specific for PKC, as revealed by using a pseudosubstrate that maintains PKC in an inactive form by occupying the active site of the enzyme (House and Kemp, 1987): indeed, incubation with the peptide inhibited both the basal and induced kinase activity (**Fig 2**). Similar results were obtained with the transformed HaCaT cell line (data not shown). These findings suggest that endogenous activation of PKC is associated with triggering the ascorbate-induced terminal differentiation program.

Ascorbate-mediated activation of PKC induces the expression of differentiation-specific genes through an AP-1-dependent pathway As almost all of the biologic markers identified in differentiating keratinocytes appear to be under the control of the AP-1 transcription factor (Welter and Eckert, 1995; Rossi *et al*, 1998), which in turn is activated by PKC, we investigated the effects of vitamin C on this transcriptional regulation. To this end, we performed EMSA on nuclear extracts derived from normal human epidermal keratinocytes grown in the presence of AA-2P or high $CaCl_2$ concentrations.

Like calcium (**Fig 3*a***, *lane 2*), ascorbate was able to enhance the ability of nuclear extracts to bind a specific oligonucleotide containing an AP-1 responsive site, both in normal human keratinocytes (**Fig 3*a***, *lane 3*) and in HaCaT cells (data not shown). This ascorbate-mediated DNA binding induction led to gene transactivation, as assessed by transient transfection studies performed with a vector containing the CAT gene under the control of the loricrin promoter, which contains AP-1 responsive sites (Welter and Eckert, 1995). Agreeing with the ability of ascorbate to induce DNA binding activity, we found that incubation with AA-2P induced a 3-fold increase of CAT activity over untreated cells, both in normal human keratinocytes and in HaCaT cells (**Fig 3*b*,** *white bars*, and data not shown). The ascorbate-induced increase of CAT activity of the wild-type loricrin promoter was completely abolished by its counterpart containing an AP-1 mutated site (**Fig 3*b*,** *gray bars*), thus suggesting that ascorbate-mediated activation of the loricrin promoter requires an intact AP-1 element.

Consistent with these results, we found that the expression of differentiation-specific genes was upregulated both in AA-2P- and Ca^{2+}-treated cells. In differentiating normal human keratinocytes (**Fig 3*c***, *lane 4*), the levels of TGase1 and loricrin transcripts were much higher than those found in control cells (compare *lanes 1 and 4*); TGase1 and loricrin expression was upregulated in a similar way in AA-2P-treated cells (**Fig 3*c***, *lane 2*). As revealed by immunoblotting analysis, the expression of the suprabasal 68 kDa K1 was also induced as keratinocytes proceeded towards terminal differentiation; thus, $CaCl_2$ treatment (1.2 mM for 5 d) increased K1 protein levels (**Fig 3*d***, *lane 4*) and vitamin C exerted a similar effect (**Fig 3*d***, *lane 2*). Although less evident, some increase in TGase3 expression was also observed (data not shown).

In accordance with our hypothesis, we found that pharmacologic down-modulation of PKC activity with GF 109203X (Toullec *et al*, 1991) partially abolished the calcium-induced, as well as the ascorbate-induced, keratinocyte differentiation through a decrease in the AP-1 DNA binding activity (**Fig 3*a***, *lanes 4, 5*). As a consequence of inhibition of AP-1 DNA binding activity, the PKC inhibitor blocked the expression of differentiation-specific genes. Indeed, GF 109203X inhibited calcium-dependent increases in TGase1 and loricrin mRNA (**Fig 3*c***, *lane 5*) as well as the calcium-dependent increase in K1 protein levels (**Fig 3*d***, *lane 5*). A similar effect on the expression of differentiation markers was also seen in ascorbate-loaded keratinocytes (**Fig 3*c***, *lane 3*, and **Fig 3*d***, *lane 3*).

The involvement of PKC in ascorbate-induced differentiation was further supported by the finding that the competitive inhibitor GF 109203X was also able to suppress the increase in CE content observed in normal human keratinocytes induced to differentiate either with 1.2 mM $CaCl_2$ or with 1 mM AA-2P (**Fig 3*e***). Comparable results were obtained with HaCaT cells grown over confluence in the presence of 1.5 mM calcium or in ascorbate-supplemented medium (data not shown).

Together, these data indicate that vitamin C can activate the epidermal differentiation program through a PKC/AP-1-dependent pathway.

Ascorbate-mediated differentiation increases vitamin C transport As the differentiation program *per se* is often accompanied by changes in capacity for antioxidant defence (Berghard *et al*, 1990; Harris, 1992), we investigated whether vitamin C transport and recycling could be modulated in keratinocytes induced to differentiate with either AA-2P or calcium.

The recent cloning of two sodium-dependent L-AA transporters, hSVCT1 and hSVCT2 (Rajan *et al*, 1999; Tsukaguchi *et al*, 1999; Wang *et al*, 1999), led us to investigate the expression of these transporters and their possible modulation during differentiation.

RT-PCR analysis performed with primers specific for either hSVCT1 or hSVCT2 (**Fig 4**) indicated that both transcripts were present in HaCaT cells (**Fig 4**, *lane 1*) as well as in normal human keratinocytes (**Fig 4**, *lane 4*), although with different abundances. Increase in extracellular calcium (**Fig 4**, *lanes 3, 6*) did not affect the expression of AA transporters; in contrast, cells grown in AA-2P-supplemented medium showed a significant increase in both transcripts, the hSVCT2 mRNA being the most affected (**Fig 4**, *lanes 2, 5*).

Increased expression of hSVCT1 or hSVCT2 was accompanied by an increased AA transport. As biochemical analyses required large amounts of biologic material, and as HaCaT cells behaved like normal human keratinocytes in terms of differentiation, under our experimental conditions, all studies on vitamin C metabolism were carried out with this cell line.

As shown in **Fig 5**, both time course (*panel a*) and dose–response (*panel b*) experiments showed that AA-2P-differentiated HaCaT cells had an increased ability to take up AA. In fact, although these cells contained a high amount of AA (about 25 mM), an increased

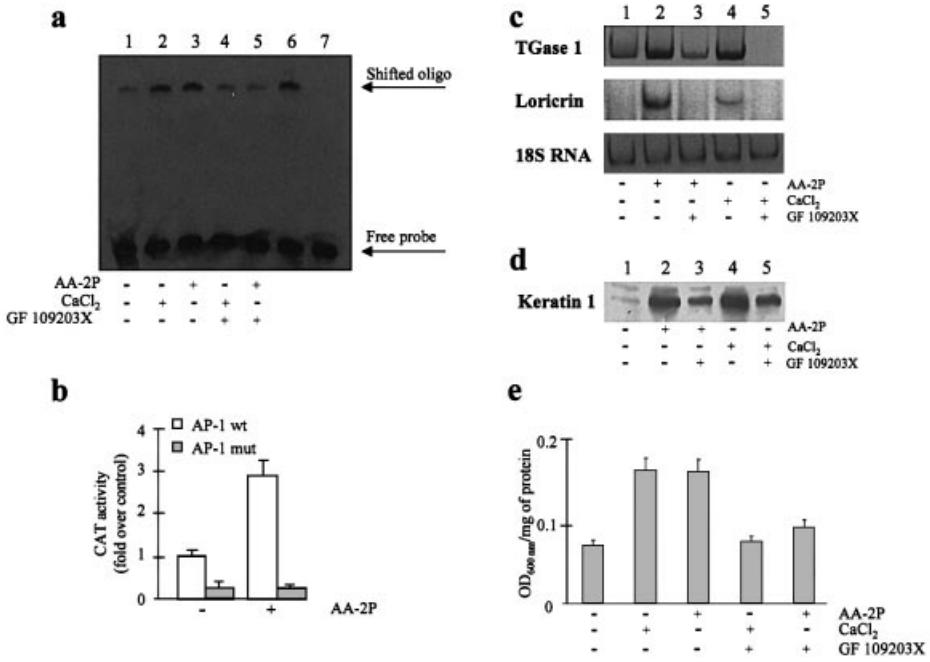

Figure 3. Ascorbate induces differentiation by regulating PKC-dependent AP-1 activity. (*a*) EMSA of nuclear extracts purified from normal human keratinocytes. Keratinocytes were grown in low calcium medium (*lane 1*), in the presence of 1.2 mM $CaCl_2$ (*lane 2*), or 1 mM AA-2P (*lane 3*) for 5 d. Samples coincubated with 0.5 μM GF 109203X are also shown (*lanes 4, 5*). Specificity controls consist of binding competition experiments of the sample shown in *lane 3* treated with a 50-fold molar excess of mutant AP-1 oligonucleotide (*lane 6*) and unlabeled consensus AP-1 oligonucleotide (*lane 7*). The radiograph is representative of three independent experiments. (*b*) CAT activity of keratinocytes transfected with an AP-1-containing promoter. Normal human keratinocyte cells were transiently transfected with the wild-type (*white bars*) and AP-1 mutated (*gray bars*) loricrin promoter CAT reporter vectors and left untreated or treated with AA-2P. Data are expressed as percentage of CAT activity over untreated cells, arbitrarily set to 100%. CAT activities were normalized by protein content and transfection efficiency (see *Materials and Methods*). Results are means ± SD of triplicate determinations carried out in three different experiments. (*c*) Analysis by RT-PCR of differentiation-specific markers in the presence of PKC inhibitor. Normal human keratinocytes were grown for 5 d in 0.05 mM Ca^{2+} (*lane 1*), 1 mM AA-2P (*lane 2*), 1 mM AA-2P plus 0.5 μM GF 109203X (*lane 3*), 1.2 mM Ca^{2+} (*lane 4*), or 1.2 mM Ca^{2+} plus 0.5 μM GF 109203X (*lane 5*). Total RNA was reverse transcribed and amplified either with TGase1 (*upper panel*) or loricrin (*middle panel*) specific primers as well as primers specific for the 18S rRNA (*lower panel*) as a loading control. Blots are representative of three independent experiments. (*d*) Western blot analysis of proteins isolated from normal human keratinocytes. Cells were grown as above and cellular extracts were purified and immunoblotted with anti-K1 antibody. The blot is representative of three similar experiments. (*e*) CE formation in the presence of PKC inhibitor. Normal human keratinocytes were grown as indicated above and CE content was assayed by spectrophotometry. Results are shown as OD600 per mg of protein. Data are the means ± SD of three independent experiments.

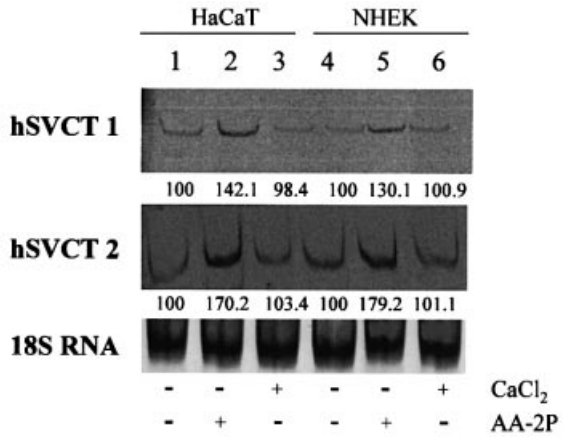

Figure 4. Ascorbate modulates the expression of sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. RT-PCR analysis of total RNA isolated from normal human keratinocyte and HaCaT cells. Cells were grown for 5 d in low (*lanes 1, 4*) or high (*lanes 3, 6*) calcium conditions, or in culture medium supplemented with 1 mM AA-2P (*lanes 2, 5*). Total RNA was reverse transcribed and amplified either with hSVCT1 (*upper panel*) or hSVCT2 (*middle panel*) specific primers; amplification of the 18S rRNA (*lower panel*) is shown as loading control. Scanning densitometry is also indicated: values are reported as fold over control, arbitrarily set to 100, after normalization with 18S rRNA. Blots are representative of four different experiments.

AA transport was measured (V_{max} of 195 pmol per min per 10^6 cells) with respect to AA-untreated, proliferating cells (V_{max} of 120 pmol per min per 10^6 cells). In contrast, Ca^{2+}-differentiated cells showed the same ability as control cells to import AA (V_{max} of 116 pmol per min per 10^6 cells). The same affinity was observed in treated and untreated cells (apparent K_m values: 213 μM for control cells *versus* 207 μM and 209 μM for AA-2P- and Ca^{2+}-differentiated cells, respectively). AA efflux was also checked, but no AA leakage was detected in our experiments. Data from kinetic analysis were consistent with the observed increase of hSVCT1 and hSVCT2 messengers only in AA-2P-differentiated cells.

We previously demonstrated that, in HaCaT cells, the oxidized form of vitamin C was also efficiently transported by facilitative glucose transporters (Savini *et al*, 2000), possibly by GLUT 1, whose expression in keratinocytes has been well documented (Gherzi *et al*, 1992). Thus, we investigated DHA uptake after differentiation induced by AA-2P or Ca^{2+}. DHA uptake reflects both its transport and reduction to AA inside the cell (Vera *et al*, 1995; Savini *et al*, 2000); we therefore carried out rapid uptake assays (5 min). This time span was chosen as the reduction process was not a rate-limiting step. As shown in time course and dose–response experiments (**Fig 5*c*,*d***), AA-2P-differentiated cells displayed almost the same kinetics of transport in comparison to control cells (apparent K_m 1.24 *vs* 1.22 mM; V_{max} 4.23 *vs* 4.39 nmol per min per 10^6 cells), whereas Ca^{2+}-induced differentiation led to a decreased transport of the oxidized form of vitamin C (apparent K_m 1.20 mM; V_{max} 1.10 nmol per min per 10^6 cells).

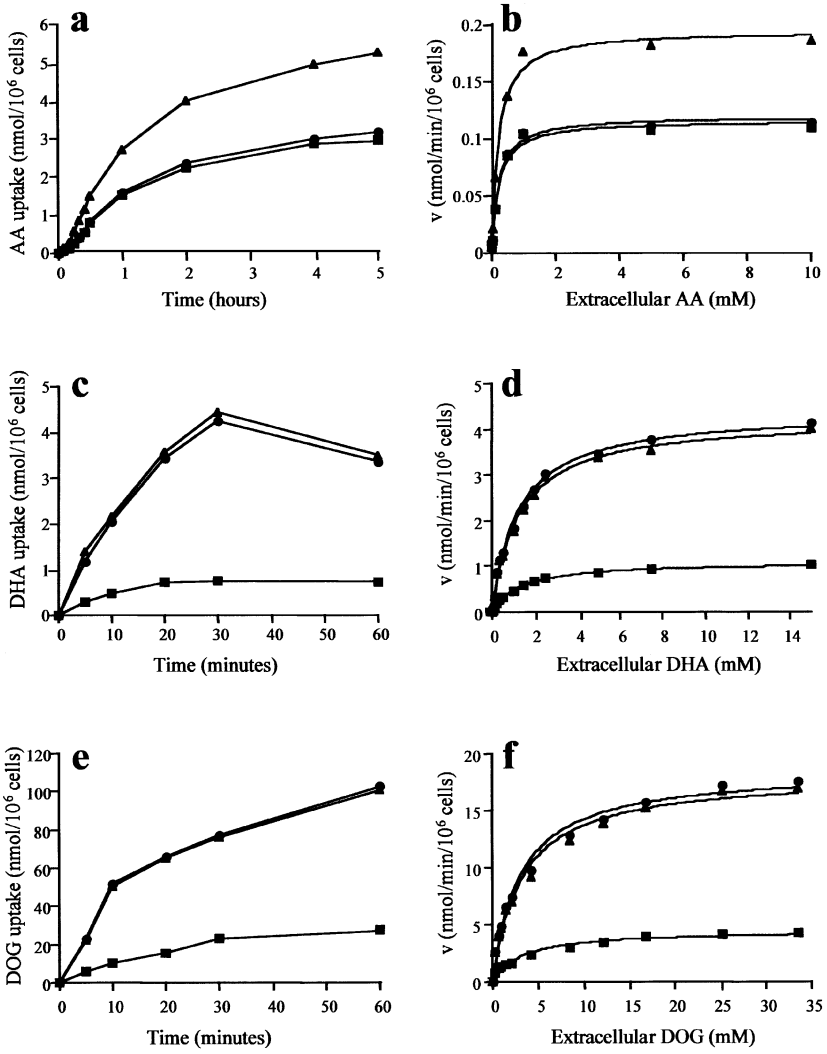

Figure 5. Ascorbate and calcium exert different effects on vitamin C uptake. (*a*) Time-dependent uptake of AA by HaCaT cells. Control cells (●) and keratinocytes differentiated either with $CaCl_2$ (■) or AA-2P (▲) were incubated with 0.2 μCi [^{14}C]-AA for the indicated times; then, AA uptake was determined by scintillation spectrometry. Values are the means of three independent experiments (SD ⩽ 7%). (*b*) Concentration dependence of AA uptake by HaCaT cells. Cells, treated as above, were incubated with [^{14}C]-AA together with the respective unlabeled compound for 2 h; then, AA uptake was determined by scintillation spectrometry. Values are the means of three independent experiments (SD ⩽ 5%). (*c*)Time-dependent uptake of DHA by HaCaT cells. Control cells (●) and keratinocytes differentiated either with $CaCl_2$ (■) or AA-2P (▲) were incubated with 0.2 μCi [^{14}C]-DHA for the indicated times; then, DHA uptake was determined by scintillation spectrometry. Values are the means of three independent experiments (SD ⩽ 4%). (*d*) Concentration dependence of DHA uptake by HaCaT cells. Cells, treated as above, were incubated with [^{14}C]-DHA together with the respective unlabeled compound for 5′; then, DHA uptake was determined by scintillation spectrometry. Values are the means of three independent experiments (SD ⩽ 7%). (*e*) Time-dependent uptake of DOG by HaCaT cells. Control cells (○) and keratinocytes differentiated either with $CaCl_2$ (□) or AA-2P (△) were incubated with 0.2 μCi [1,2-^{3}H]-2-DOG for the indicated times; then, DOG uptake was determined by scintillation spectrometry. Values are the means of three independent experiments (SD ⩽ 6%). (*f*) Concentration dependence of DOG uptake by HaCaT cells. Cells were incubated with [1,2-^{3}H]-DOG together with the respective unlabeled compound for 5′; then, DOG uptake was determined by scintillation spectrometry. Values are the means of three independent experiments (SD ⩽ 6%).

The activity of transporters was checked by measuring the transport of deoxyglucose (which is accumulated as deoxyglucose-6-phosphate). As shown in **Fig 5** (*panels e, f*), the decrease in deoxyglucose transport paralleled that observed for DHA: this observation was consistent with the reported down-modulation of GLUT1 expression by calcium in keratinocytes (Gherzi *et al*, 1992).

Preliminary results obtained with normal human keratinocytes suggest that these data could also be extrapolated to primary cultures (data not shown).

Altogether, these results indicate that AA-induced differentiation was characterized by an improved ability to import vitamin C, both by increasing AA transport and by maintaining basal DHA transport, which was otherwise inhibited by calcium-triggered differentiation.

Ascorbate-mediated differentiation modulates vitamin C recycling Once inside the cell, vitamin C is maintained in the reduced form by different enzymatic systems; therefore, we addressed whether differentiation induced changes in the enzymatic activities involved in vitamin C recycling.

AA-2P-induced differentiation led to a decreased intracellular AFR-reductase activity in HaCaT cells (53% ± 3%), whereas transmembrane AFR-reductase and DHA-reductase activities were not affected (**Table I**). On the other hand, calcium-mediated differentiation was accompanied by increased intracellular (180% ± 10%) and transmembrane AFR-reductase (138% ± 5%) activities with respect to untreated cells (**Table I**). At the same time, enzymatic DHA reduction was decreased with respect to proliferating cells (76% ± 4%), with the NADPH/GSH-dependent activity being the most affected (46% ± 2%) and the thioredoxin and NADH-lipoic-acid-dependent reductases slightly affected. Moreover, we found that total glutathione content of calcium-treated cells was drastically decreased (37% ± 2%) in comparison with untreated cells; in contrast, AA-2P-differentiated cells showed increased intracellular GSH levels (150% ± 8%) with respect to control cells.

DISCUSSION

AA plays an important role in epithelial cells as a protective agent against oxidative stress and promoter of the wound healing process. In this study, we have demonstrated that vitamin C is a direct modulator of keratinocyte differentiation, and that ascorbate-triggered differentiation proceeds through a PKC-dependent activation of the AP-1 transcription factor.

Cultured keratinocytes are induced to differentiate in response to several extracellular stimuli, among which calcium plays a key role (Hennings *et al*, 1980). Ca^{2+}-mediated events leading to keratinocyte maturation are regulated by distinct AP-1 binding complexes (Rutberg *et al*, 1996; Rossi *et al*, 1998). Antibody staining studies

Table I. Ascorbate and calcium exert different effects on vitamin C recycling in HaCaT cells

	DHA-reductase activities				AFR-reductase activities	
Sample	Basal DHA-reductase[a] (%)	NADPH GSH-dependent DHA-reductase[b] (%)	NADH-lipoic acid-dependent DHA-reductase[c] (%)	Thioredoxin reductase[d] (%)	Intracellular AFR reductase[d] (%)	Trans membrane AFR reductase[d] (%)
Ctrl	100	100	100	100	100	100
CaCl2	76	46	98	94	180	138
AA-2P	106	105	95	118	53	101

[a]Corrections were made for non-enzymatic reduction of DHA by a concentration of GSH corresponding to its cellular content (2–4% of the enzymatic activity). The basal reaction mixture was composed of 50 mM Tris/HCl, pH 7.5, containing 1 mM DHA, 1 mM EDTA and 0.2 mg of cell homogenate protein.
[b]NADPH-GSH-dependent DHA-reductase activity was measured in the presence of 0.4 mM NADPH + 2 mM GSH.
[c]NADH-lipoic acid-dependent DHA-reductase activity was measured in the presence of 0.5 mM lipoic acid + 0.4 mM NADH.
[d]Thioredoxin and AFR reductase activities were measured as described in *Materials and Methods*. Corrections for blanks were made when appropriate. The table reports the percentage with respect to the control, arbitarily set to 100%. Data are the mean of three independent experiments, each one performed in triplicate (SD ≤6%).

have demonstrated that, in the spinous layer of the epidermis, AP-1 heterodimers consist mainly of Fra-1 and JunD, whereas Fra-2 and JunB complexes predominate in the granular layer (Welter and Eckert, 1995). In differentiating cells, calcium increases AP-1 DNA binding activity and affects the composition of AP-1 complexes via obligatory activation of PKC (Rutberg *et al*, 1996). We found that, like calcium, AA stimulated the expression of genes associated with a differentiated phenotype, such as suprabasal keratins (K1), TGases (TGase1 and TGase3), and loricrin, both in primary and transformed keratinocytes. Vitamin C is also necessary for the improvement of the lipid profile, and thus the barrier formation of the stratum corneum, in reconstructed human epidermis (Ponec *et al*, 1997). These findings reveal a new role for vitamin C in skin biology as an inducer of differentiation.

The conversion of basal keratinocytes to a more differentiated phenotype, characterized by the formation of CE, was mediated through the ascorbate-induced activation of AP-1. Indeed, we have previously shown that vitamin C regulated AP-1 activity by modulating fra-1 expression and by inhibiting the JNK-mediated phosphorylation of c-Jun (Catani *et al*, 2001); thus, the altered composition of AP-1 dimers may specify the differentiation-dependent pattern of gene expression. AP-1 is a crucial target for redox modulators both in keratinocytes (Rossi *et al*, 2000) and in other models (Melino *et al*, 1997, 2000). Furthermore, we demonstrated that AA activated PKC by translocating it from the cytosol to the membrane and that pharmacologic down-modulation of PKC in differentiating keratinocytes partially abolished the ascorbate-induced AP-1 DNA binding activity and the expression of AP-1-targeted genes. The induction of PKC is of particular relevance, considering that this enzyme contains redox-sensitive cysteine residues both in regulatory and catalytic domains (Gopalakrishna and Jaken, 2000). The availability of large amounts of AA may activate PKC either by relieving its autoinhibition via the regulatory domain or by preserving the free sulfydryls needed for catalytic activity.

Although both AA and Ca^{2+} act as differentiating agents in a similar way, nonetheless we report here that, at the end of differentiation, keratinocytes were characterized by different vitamin C homeostasis and glutathione levels. Exposure to high levels of exogenous AA increased cellular efficiency of transport systems rather than recycling ability. Our results demonstrated that AA transport was achieved through an enhanced expression of the transporters hSVCT1 and hSVCT2. It is noteworthy that keratinocytes expressed both systems, whereas a specific distribution of the two transporters has been reported in mammalian tissues, with hSVCT1 being expressed in intestine, kidney, and liver, and hSVCT2 transcripts being ubiquitous (Tsukaguchi *et al*, 1999). In our model, both transporters appear to contribute to AA accumulation inside the cell: nonetheless, further studies are necessary to clearly delineate the physiologic relevance of these two transporters in skin and to establish which transporter activity predominates *in vivo*.

Furthermore, AA-mediated differentiation increased intracellular glutathione levels, helping cells to maintain a well-balanced antioxidant status, whereas Ca^{2+}-induced differentiation induced glutathione deficiency. Thus, we suggest that Ca^{2+}-differentiated keratinocytes must increase ascorbate utilization in order to compensate for intracellular glutathione depletion and maintain the redox balance; consequently, although they were less efficient in DHA transport and reduction, they were better able to recycle both intracellular and extracellular AFR than proliferating cells. Transmembrane AFR-reductase activity has been shown to be modulated also in TPA-induced differentiation of HL-60 cells (Buron *et al*, 1993); moreover, it has been described previously that other antioxidant enzymes showed enhanced expression in glutathione-depleted cells (Salvemini *et al*, 1999). In contrast, ascorbate-mediated differentiation led to a reduced AFR-reductase activity showing that this enzymatic activity was regulated by redox conditions.

In conclusion, we have shown that vitamin C has a novel signaling function, being able to trigger skin differentiation and to overcome the differentiation-dependent oxidative stress. Thus, vitamin C might be employed as a drug permitting faster recovery of skin integrity and strength during wound healing, as it (i) regulates epidermal differentiation via activation of PKC, which is itself a modulator of wound healing (Chandrasekher *et al*, 1998), and (ii) protects keratinocytes against oxidative stress, particularly during the inflammatory phase of wound repair, via improvement of the hydrophilic antioxidant status inside cells.

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