Topically applied lactic acid increases spontaneous secretion of vascular endothelial growth factor by human reconstructed epidermis

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Summary

Background   Alpha-hydroxy acids (AHAs) are widely used for the treatment of hyperkeratotic skin disorders and photodamaged skin.

Objectives   To investigate the effect of lactic acid (LA) on the secretion of cytokines by keratinocytes (KCs) of human reconstructed epidermis.

Methods   Creams containing 1·5%, 3% or 5% LA or vehicle controls were topically applied on to human epidermal equivalents (EEs). After 24 h, EEs were analysed for morphology and for the presence of apoptotic cells. Secretion of vascular endothelial growth factor (VEGF), angiogenin (ANG) and interleukin (IL)-8 was measured in the supernatants by enzyme-linked immunosorbent assay.

Results   LA led to a concentration-dependent increase in apoptotic cells as determined by cell morphology and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling. VEGF secretion was increased 2·5- and 2·8-fold ($P < 0·05$) over vehicle control after treatment with 1·5% and 3% LA, respectively. No significant increase in VEGF secretion was detected with 5% LA. In contrast to VEGF, secretion of ANG was decreased by LA in a concentration-dependent manner (0·5-fold for 5% LA; $P < 0·01$). No significant changes in IL-8 secretion were found with any of the concentrations tested.

Conclusions   Our data demonstrate that the topical application of AHAs modulates the secretion of cytokines by KCs. Regulation of KC-derived growth factors and cytokines by AHAs might represent a mechanism contributing to their therapeutic effects in disorders such as photoageing.

Key words: alpha-hydroxy acids, angiogenesis, lactic acid, skin equivalent, vascular endothelial growth factor

Alpha-hydroxy acids (AHAs) comprise a group of organic acids naturally found in foods such as sugar cane (glycolic acid), sour milk (lactic acid), apples (malic acid) or citrus fruits (citric acid).[1] They have been used to treat skin disorders associated with hyperkeratinization, including ichthyosis, acne and dry skin.[1,2] Topical treatment of these conditions with AHAs at concentrations of 5–20% leads to exfoliation of the stratum corneum due to diminished corneocyte cohesion, resulting in a more flexible stratum corneum.[1] Application of high concentrations of AHAs (70–80%) leads to epidermolysis and is commonly used for superficial skin peeling and treatment of actinic and seborrhoeic keratosis.[3]

Recently, AHA-containing formulations have also been used for the treatment of photoaged skin.[4] Clinically, photoaged skin is characterized by laxity, roughness, sallowness and irregular hyperpigmentation.[4] Skin vessels are also affected and may be completely obliterated, telangiectatic and tortuous. The horizontal plexus and the subepidermal capillary loops are generally diminished.[5]

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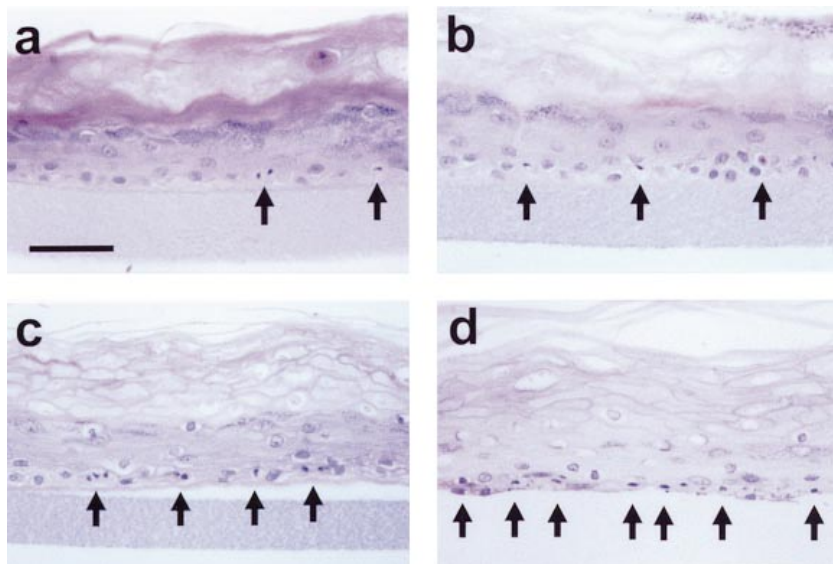
Figure 1. Samples were processed for histology 24 h after topical application. As compared with vehicle treatment (a), morphological changes and increased cell death were observed after treatment with lactic acid-containing creams at increasing concentrations: (b) 1·5%; (c) 3%; (d) 5%. Arrows mark apoptotic cells (haematoxylin and eosin; bar = 50 μm).

Topical use of AHAs has been reported to reduce overall severity of photodamage.[6,7] Histologically, structural changes of both the epidermis and the dermis have been reported after AHA application. These changes comprise an increased thickness of the viable epidermis,[8-10] increased deposition of hyaluronic acid,[8] reduced basal cell atypia and a more undulating rete pattern. In the dermis, increased production of acid mucopolysaccharides,[9] hyaluronic acid and chondroitin sulphate,[8] as well as an increased density of collagen fibres and less fragmented, irregular elastic fibres,[9] have been reported.

There are only a few studies addressing the mechanisms by which AHAs may induce these profound structural changes.[11] As AHAs have recently been shown to prevent the regression of skin vessels due to corticosteroids,[10] we investigated the effects of AHAs on the secretion of the angiogenic cytokines vascular endothelial growth factor (VEGF), interleukin (IL)-8 and angiogenin (ANG) by keratinocytes (KCs).

Materials and methods

Epidermal equivalents

Human epidermal equivalents (EEs) grown in collagen-coated cell culture inserts without fibroblasts under
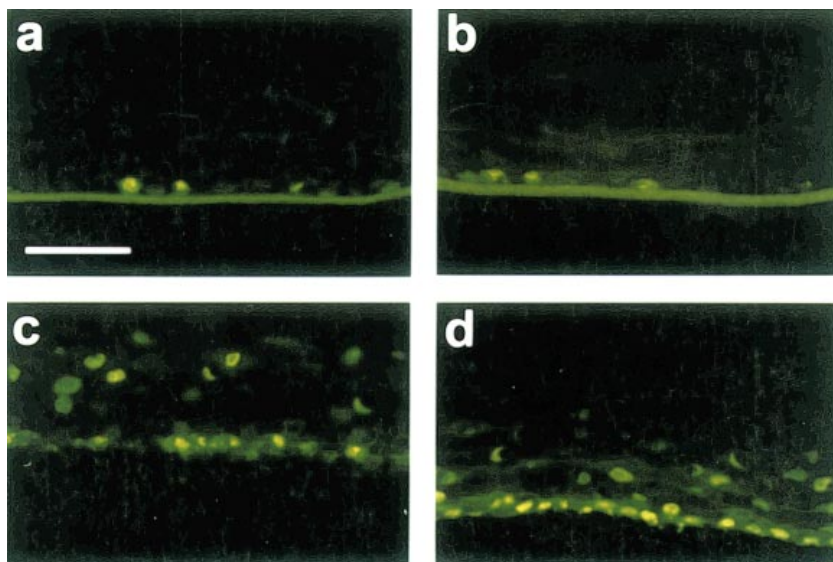
Figure 2. Cell death after lactic acid (LA) treatment was caused by apoptosis, as confirmed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling performed 24 h after application of vehicle (a) or creams containing 1·5% (b), 3% (c) or 5% (d) LA. Apoptotic cells are stained bright yellowish-green (bar = 50 μm).

serum-free conditions (EpiDerm™) were obtained from Mattek Corporation (Ashland, MA, U.S.A.). They represent an *in vitro* reconstructed model for differentiating human epidermis. Individual EE samples have a diameter of 8 mm. EEs were shipped by the manufacturer at 4 °C. Upon arrival, the cell culture inserts were changed from the shipment support into six-well plates containing 900 μL of prewarmed serum-free growth medium provided by the manufacturer. After 2–3 h, medium was changed to 900 μL Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Vienna, Austria) without growth supplements and cultured overnight, after which the topical formulations were applied (see below). For all experiments EEs were kept at the air–liquid interface at 37 °C in a 5% CO_2/95% air environment in a humidified incubator.

Composition of the creams

The creams used in this study were obtained from the Centre de Recherches et Investigation Epidermiques et Sensorielles (Neuilly, France). Lactic acid (LA) was added to the vehicle at 1·5%, 3% and 5% v/v (Table 1) and the pH was adjusted to 4·3. The osmolarity of the vehicle was 73 mmol L^{-1} and increased to 321, 563 and 881 mmol L^{-1} with 1·5%, 3% and 5% LA, respectively (where approximately 300 mmol L^{-1} is that of plasma and of the culture medium DMEM). Because addition of LA resulted in a higher fluidity of the creams, viscosity of the different formulations was adjusted to the range of 798–966 mPa s^{-1} by adding increasing concentrations of sepigel 305 (Seppic-Montanoir, Paris, France) (Table 1). As addition of comparable amounts of sepigel 305 to vehicle alone resulted in a strong increase in the viscosity (not shown), vehicle containing 1·5% sepigel 305 was used as a control for all experiments.

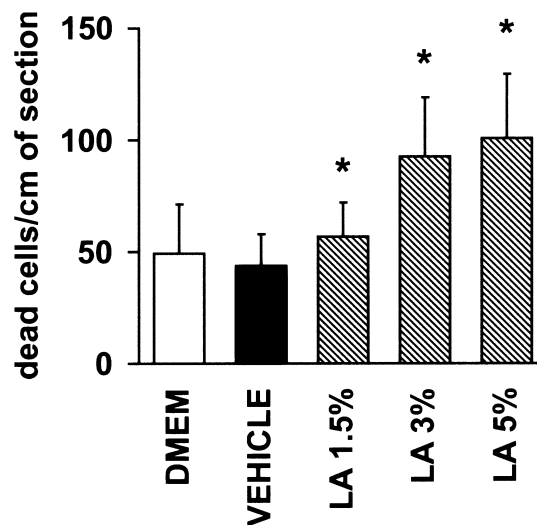**Table 1.** Composition of lactic acid (LA) -containing creams and controls

|	Vehicle	LA 1·5%	LA 3%	LA 5%
LA (%)	0	1·5	3·0	5·0
Sepigel 305 (%)	1·5	3·5	4·0	5·0
0·5 mol L^{-1} HCl (%)	0·31	0	0	0
pH	4·3	4·3	4·3	4·3
Viscosity (mPa s^{-1})	840	966	798	966

Stimulation experiments

After overnight culture of EEs, medium was replaced with 900 μL of fresh DMEM, and creams containing different concentrations of LA or vehicle were applied in 50-μL aliquots directly on to the stratum corneum with a multipipette (Eppendorf, Hamburg, Germany). After 24 or 48 h, supernatants were collected, centrifuged at 17,600 *g* and stored at − 70 °C until further analysis. At the same time the EEs, together with the membranes of the cell culture inserts, were cut out, formalin fixed and paraffin embedded. The morphology of EEs was then evaluated by haematoxylin and eosin (H&E) staining of 4-μm sections. Six independent experiments were performed in triplicate. One experiment was excluded from analysis because of inconclusive triplicate determinations of control samples.

Cytokine immunoassays

Cell culture supernatants were analysed for different cytokines using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions: VEGF (CYTElisa-VEGF, CYTimmune Sciences, College Park, MD, U.S.A.),

Figure 3. Application of lactic acid (LA)-containing creams significantly increased the number of apoptotic cells in human epidermal equivalents. Apoptotic cells were identified by morphological criteria in haematoxylin and eosin-stained sections and counted independently by two blinded investigators. Counts were related to the length of the sections examined and given as an apoptotic index. Results are shown as mean ± SD from five independent experiments. DMEM, Dulbecco's modified Eagle's medium; *$P < 0.01$.
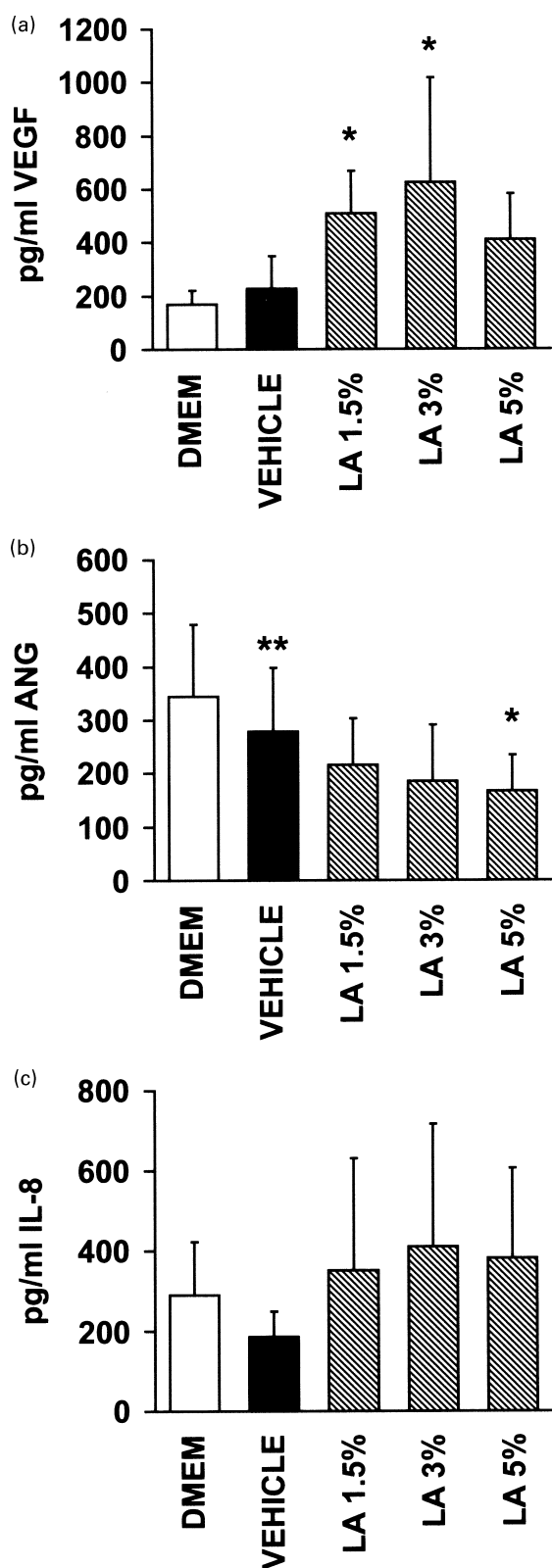
ANG (R&D Systems, Minneapolis, MN, U.S.A.) and IL-8 (Biosource International, Camarillo, CA, U.S.A.).

Detection of apoptotic cells

Apoptotic cells were identified on H&E-stained sections by the characteristics of apoptosis, i.e. cell shrinkage with condensed, pyknotic nuclei and eosinophilic cytoplasm. Apoptotic cells were counted by two blinded investigators (M.R., C.M.) and an apoptotic index (AI) was calculated (= number of apoptotic cells per cm section). The morphological data were confirmed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) with the '*in situ* cell death detection kit' (Boehringer Mannheim, Vienna, Austria) according to the manufacturer's instructions.

Statistical analysis

To compare the different treatment groups Student's *t*-tests were calculated using the median values of the ELISA triplicate determinations or the mean of the AI. Results were considered significant at $P < 0.05$.

Results

Effects of lactic acid treatment on the viability of keratinocytes

In a first set of experiments, samples were histologically analysed 24 and 48 h after application of the respective creams. After 48 h the morphology of EEs cultured in DMEM without creams deteriorated, i.e. EEs comprised only two or three instead of four or five layers of nucleated cells (not shown). Therefore, all further experiments were performed for 24 h only. After 24 h, treatment with LA-containing creams led to morphological changes of EEs in a concentration-dependent manner (Fig. 1b–d): the stratum corneum appeared swollen and showed a decreased staining intensity and

Figure 4. Topically applied lactic acid (LA) differentially modulated vascular endothelial growth factor (VEGF), angiogenin (ANG) and interleukin (IL)-8 secretion by human epidermal equivalents. Twenty-four hours after application of vehicle or LA-containing creams, supernatants were analysed for the presence of VEGF (a), ANG (b) and IL-8 (c) by enzyme-linked immunosorbent assay. Results are shown as mean ± SD from five independent experiments [a: *$P < 0.05$, LA compared with vehicle; b: *$P = 0.01$, LA compared with vehicle; **$P = 0.02$, vehicle compared with Dulbecco's modified Eagle's medium (DMEM)].

the stratum granulosum disappeared. The number of apoptotic cells increased with increasing LA concentration (Fig. 1a–d, arrows). The apoptotic cells were preferentially located in the basal KC layers. To confirm that these cells underwent apoptosis, we performed TUNEL staining. As depicted in Figure 2(a–d), distribution and location of TUNEL-positive, bright yellowish-green nuclei corresponded to apoptotic cells identified by H&E staining. A significant increase in the AI was observed after treatment with all concentrations of LA when compared with vehicle alone, i.e. 1·4-, 2·0- and 2·4-fold with 1·5%, 3% and 5% LA, respectively (Fig. 3).

Application of lactic acid has different modulatory effects on vascular endothelial growth factor, angiogenin and interleukin-8 secretion by keratinocytes

The supernatants of five independent experiments performed in triplicate were analysed for levels of VEGF, ANG and IL-8. Without application of creams, secretion of VEGF by EEs cultured in DMEM alone ranged from 87 to 236 pg mL^{-1} (mean ± SD 157 ± 53) after 24 h (Fig. 4a). Application of vehicle had no significant ($P = 0·21$) effect on VEGF secretion as compared with DMEM (Fig. 4a, vehicle: mean ± SD 225 ± 123 pg mL^{-1}). By contrast, treatment with creams containing 1·5% and 3% LA increased spontaneous mean ± SD VEGF secretion 2·5 ± 0·6 fold ($P = 0·02$) and 2·8 ± 0·9 fold ($P = 0·045$), respectively (Fig. 4a, LA 1·5%: 508 ± 157 pg mL^{-1}; LA 3%: 625 ± 390 pg mL^{-1}). Application of 5% LA did not significantly influence mean ± SD VEGF secretion (2·1 ± 1·8 fold, $P = 0·96$; Fig. 4a, LA 5%: 412 ± 169 pg mL^{-1}).

The baseline level of ANG release by EEs ranged from 120 to 478 pg mL^{-1} (mean ± SD 344 ± 135; Fig. 4b). Application of vehicle significantly reduced ($P = 0·02$; Fig. 4b, vehicle: mean ± SD 279 ± 119 pg mL^{-1}) and treatment with LA-containing creams further decreased mean ± SD ANG release (Fig. 4b, LA 1·5%: 215 ± 87 pg mL^{-1}; LA 3%: 185 ± 105 pg mL^{-1}; LA 5%: 167 ± 68 pg mL^{-1}) as compared with vehicle, reaching significance with 5% LA ($P = 0·01$).

The baseline level of IL-8 release by EEs ranged from 151 to 499 pg mL^{-1} (mean ± SD 290 ± 132; Fig. 4c). Changes in mean ± SD IL-8 release after treatment with LA-containing creams strongly varied between experiments, leading to a modest increase that did not reach statistical significance (Fig. 4c, LA 1·5%:

350 ± 280 pg mL^{-1}; LA 3%: 411 ± 307 pg mL^{-1}; LA 5%: 381 ± 224 pg mL^{-1}).

Discussion

AHAs have been used for the treatment of various skin disorders, including photodamaged skin.[3,4] In the present study we hypothesized that some of the effects of AHAs are due to their action on KCs and that changes observed in the dermis after AHA treatment[6–9] might be mediated by KC-derived soluble factors; therefore, we investigated the effect of topical AHA application on the spontaneous secretion of the angiogenic cytokines VEGF, ANG and IL-8 by human *in vitro* reconstructed epidermis.

AHA application differentially modulated the secretion of these three cytokines. The release of VEGF by EEs was significantly increased after treatment with creams containing 1·5% and 3% but not 5% LA, whereas the release of IL-8 was not significantly altered and that of ANG decreased with increasing concentrations of LA.

AHA application clearly showed a toxic effect on the EEs, in that increasing concentrations of LA led to increased apoptotic cell death of KCs as judged by morphology and TUNEL staining. We can exclude the possibility that this effect was due to pH differences of the culture medium because the vehicle control was adjusted to the same pH as AHA-containing creams. Regarding the influence of the osmolarity of the different creams on cell death, we cannot exclude that the higher osmolarity of the 3% and 5% LA-containing creams (see Materials and methods) contributed to the induction of cell death. However, vehicle alone (which was hyposomolar as compared with culture medium) neither increased cell death nor induced a deterioration of the epidermal architecture, suggesting that the contribution of variation of osmolarity to cell death is slight. Induction of cell death was strongest at 5% LA, where most of the KCs of the EEs were dead. *In vivo*, much higher concentrations of AHAs are regularly used to obtain therapeutic effects without obvious damage to the epidermis.[3] Only when applied at concentrations as high as 70%, as used for peeling procedures, are irritating and damaging effects to the skin observed.[12] The difference between *in vitro* and *in vivo* treatment is probably due to a combination of factors. Although *in vitro* differentiated EEs have a fully developed and functional stratum corneum,[13–15] their barrier function is reportedly inferior to the epidermal barrier *in vivo*. Therefore, the amount of LA

that reaches the KCs is probably much higher than in the epidermis *in vivo.* Moreover, *in vivo,* toxic substances and metabolic products are removed by the skin microcirculation, a mechanism lacking in the *in vitro* model. As a result, toxic metabolites might accumulate in EEs after AHA treatment and ultimately account for KC apoptosis. These limits of the model have to be taken into account when extrapolating conclusions from data obtained *in vitro* to the *in vivo* situation.

VEGF is expressed by KCs in human epidermis[16,17] and is produced by KCs in monolayer culture.[18] It is regulated by a variety of factors including hypoxia,[19] cytokines,[20] sensitizing and non-sensitizing irritants,[21] and retinoids.[22] Here we report for the first time the regulation of VEGF production by KCs in epidermal organotypic culture. In this system application of LA-containing creams strongly increased the release of VEGF. This increase was due to *de novo* protein synthesis rather than to the release of preformed protein by dying cells, because in normal EEs only a small proportion of the VEGF found in the supernatant was detected within lysates of EEs (data not shown). Moreover, no upregulation was found with 5% LA, which gave the strongest induction of apoptotic cell death. We do not know at present whether the observed upregulation of VEGF is caused by autocrine or paracrine mechanisms involving other KC-derived cytokines or by the direct action of LA. The exact pathway leading to the induction of VEGF secretion by KCs in EEs remains to be explored.

With regard to the implications of the finding that VEGF production by KCs is upregulated by AHA treatment, alterations of cutaneous vessels are commonly observed in chronic photodamage,[5] and VEGF has major effects on endothelial cells.[23,24] It stimulates endothelial cells to proliferate,[25] migrate[26] and express genes involved in angiogenesis,[27] resulting in the formation of new blood vessels. Besides its angiogenic properties, VEGF has recently also been reported to act as a potent survival factor for endothelial cells.[28–30] An increased production of VEGF by AHAs *in vivo* could, therefore, exert a beneficial effect on the skin microvasculature by both helping to maintain it and by inducing the formation of new vessels. A precedent for such an effect exists: Lavker *et al.* recently showed that topical AHA treatment can prevent the regression of skin microvessels caused by topical corticosteroid treatment and that it appears to increase skin vascularization.[10] It is very likely that the induction of KC-derived VEGF plays a part in the effect observed by these authors.

Both other cytokines studied, i.e. IL-8 and ANG, exhibit angiogenic activity *in vivo* and mitogenic effects on endothelial cells *in vitro.*[31–34] In this study they were, like VEGF, constitutively secreted by EEs. ANG is expressed by a variety of cell types,[35,36] and this is the first demonstration that it is produced by human KCs. The biological relevance of its production by KCs remains to be determined. Whereas the production of IL-8 was not regulated by AHA treatment, the release of ANG continuously decreased with increasing concentrations of LA, coinciding with increasing cell death, which might reflect the inability of EEs to maintain baseline ANG production due to increased apoptosis rather than to active downregulation.

In summary, we have demonstrated that topical application of AHAs can modulate the production of cytokines by KCs. We suggest that the increased release of VEGF by KCs after AHA treatment of photodamaged skin may help to halt the regression of skin vessels and lead to neoangiogenesis, thereby contributing to the therapeutic effects observed with these substances. Whether or not topical AHAs induce the production of other cytokines and/or enzymes by KCs will be an interesting area of research in the future.

Acknowledgments

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