Effects of Ascorbic Acid on Keratinocyte and Epidermalization of Skin

Kyoung-Chan Park, M.D., Hye-Ryung Choi, M.S., Hyun-Joo Cho, M.S., Soon-Kyum Kim, Sun-Bang Kwon, Ph.D., Dong-Seok Kim, Ph.D., Young-Joon Cho, M.D., Sang-Woong Yoon, M.D.

Department of Dermatology, Bundang Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea

Background: There are different models of skin substitutes, but no skin substitutes have the characteristics of native skin. It was reported that the incubation of skin substitutes in medium containing ascorbic acid extends cellular viability and promotes formation of an epidermal barrier *in vitro*.

Objective: The purpose of this study is to observe the effects of ascorbic acid on the proliferation of keratinocytes and on the reconstruction of epidermis.

Materials and Methods: Normal human keratinocytes and fibroblasts were isolated and used for culturing living skin equivalent (LSE).

Results: When ascorbic acid was added, the expression of p63 and α6 integrin was definitely increased compared to control models. In addition, ascorbic acid increased the proliferation of normal human keratinocytes at a dose dependent manner. Especially, ascorbic acid induced the phosphorylation of ERK and up-regulation of EGF-R.

Conclusion: Results suggest that ascorbic acid is essential in the control of keratinocyte proliferation and basement membrane formation. Ascorbic acid-related keratinocytes proliferation is seemed to be mediated by ERK phosphorylation and EGF-R up-regulation. (Ann Dermatol 16(2) 45~51, 2004)

Key Words: Ascorbic acid, Keratinocytes, LSE (living skin equivalent)

Living skin equivalent (LSE) have become useful as adjunctive treatments for excised, full-thickness burns.[1] There are different models of skin substitutes but no LSE have the anatomy and physiology of native skin. Ascorbic acid is known to increase the biosynthesis of collagen and incubation of LSE in ascorbic acid-containing medium promotes the formation of epidermal barrier *in vitro*, and promotes engraftment[1,2]. However, molecular mechanism of these findings is not clarified yet. We observed that incubation of LSE in ascorbic acid-containing medium facilitates the formation of LSE with thick epidermis, compact horny layer, and more regular arrangement of basal cells. Based on these observations, we investigated the effects of ascorbic acid in the reconstruction of LSE. Furthermore, we studied the effect of ascorbic acid on cultured normal human keratinocytes and the mechanism of these proliferative effects.

Received January 10, 2004
Accepted for publication April 10, 2004

Reprint request to: Kyoung-Chan Park, M.D., Department of Dermatology, Bundang Seoul National University Hospital, Seoul National University College of Medicine, 300 Gumi-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-707, Korea
Tel. 82-031-787-7439, Fax: 82-2-3675-1187
E-mail. gcpark@snu.ac.kr

MATERIALS AND METHODS

Primary culture of keratinocytes and fibroblasts

Human foreskin was obtained during circumcision and normal human keratinocytes were isolated as

previously described, and were cultured in keratinocyte growth medium (KGM, Clonetics, SanDiego, CA)[3,4]. Human fibroblasts were obtained from the dermal parts of the samples and cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Reconstruction of skin equivalents

Dermal substitutes were prepared according to the method of Bell, with some modifications[5,6]. Type I collagen was extracted from rat-tails. The concentration of collagen was measured using Bio-Rad protein assay reagent (Bio-Rad Lab., Hercules, CA) and diluted to 0.5 mg/mL in 0.1% acetic acid solution. To construct the fibroblast embedded collagen gels, cultured fibroblasts were suspended in collagen solution, which was made by mixing eight volumes of type I collagen solution with one volume of 10X concentrated DMEM and one volume of neutralizing buffer (0.05 N NaOH, 0.26 mM $NaHCO_3$, and 200 mM HEPES). The mixture was transferred to a millicell (Millipore Corp, Bedford, MA), and allowed to gel for 15 min. Cultured keratinocytes (1×10^6/millicell) were seeded in the 3 : 1 mixture of DMEM and Ham's nutrient F12 medium, and incubated while submerged for 7 days, and at the air-liquid interface for further 7 days. During the air exposure period, ascorbic acid was added at the concentration of 25 μg/mL.

Histology and immunohistochemistry

At the end of the growth period, samples were fixed with Carnoy's fixative and embedded in paraffin. Specimens of the unexposed skin from healthy volunteers were used as a control. Immunohistochemical studies were performed on deparaffinized sections using the avidin-biotin-peroxidase complex technique (DAKO, Carpinteria, CA). Antibodies used in this study were as follows: a monoclonal antibody against involucrin (I9018, Sigma, St. Louis, MO); a monoclonal antibody against filaggrin (BT576, Biomedical Technologies, Inc., Stoughton, MA); a monoclonal antibody against p63 (sc-8431, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); a monoclonal antibody against proliferating cell nuclear antigen (PCNA; M0879, DAKO, Glostrup, Denmark); a monoclonal antibody against Ki67 (0505, Immunotech, Marseille, France); a polyclonal antibody against α_6 integrin (sc-6597, Santa Cruz Biotechnology, Inc.); and a polyclonal antibody against epidermal growth factor receptor (EGFR; sc-03, Santa Cruz Biotechnology, Inc.). At each staining, normal goat IgG (AB-108-C, R&D systems, Inc.), rabbit IgG (AB-105-C, R&D systems, Inc.), or mouse IgG1 isotype control (MAB002, R&D systems, Inc.) was used as negative control.

MTT assay for cell proliferation

Cells (4×10^4 cells/well) were seeded into 24-well plates. After serum starvation for 24 h, the cells were incubated with ascorbic acid for indicated times at 37℃ in 5% CO_2. After adding 100 μL/well of MTT solution (5 mg/mL), the plates were incubated for another 4 h. Supernatants were removed and the formazan crystals were solubilized in 1 mL of dimethylsulfoxide. Optical density was determined at 540 nm using an ELISA reader (TECAN, Salzburg, Austria).

Western blot analysis

Cells were grown in 100 mm culture dishes, starved of serum for 24 h, and treated with test substances. The cells were then lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF-membranes, which were then saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, U.K.).

Statistics

Differences between results were assessed for significance using the Student's *t*-test.

RESULTS

Reconstruction of LSE and the effects of ascorbic acid

Human skin was reconstructed on fibroblast populated type I collagen gel. Characteristic multi-

layering and stratification of epidermis was observed. Addition of ascorbic acid induced significant differences compared to control model (LSE CTL, Fig. 1). Generally, epidermis became thick and basal layer looked more compact (LSE Vit. C, Fig. 1).

Effects of ascorbic acid on differentiation and proliferation markers

Involucrin is a protein precursor of the epidermal cornified envelope[7]. In normal skin only the upper spinous and granular layers expressed involucrin (Fig. 1). However, entire epidermis in the LSE CTL model showed intense staining (Fig. 1). In ascorbic acid treated model, lower part of epidermis was not stained and this pattern seemed to be more similar to that of normal skin. Profilaggrin, proteolytically processed into individual domains during terminal differentiation, is a keratinocyte specific phosphoprotein expressed in the granular layer[8]. In normal skin, filaggrin was stained only in the granular layer (Fig. 1). A similar staining pattern was observed in both (LSE CTL) model and (LSE Vit. C) model (Fig. 1). PCNA is present through out the cell cycle in proliferating cells[9]. In normal skin, PCNA expression was mainly observed in the basal layer of epidermis (Fig. 2). In both LSE models, PCNA positive cells were observed mainly at suprabasal areas and there are no significant differences (Fig. 2). Ki67, a nuclear protein that is expressed in proliferating cells, was also observed in the basal layer of epidermis[10]. There are no significant differences in Ki67 expression in these models (Fig. 2). p63, a member of the p53 gene family, is a specific marker of human corneal and epidermal stem cell. p63 is expressed in basal/suprabasal cells of epidermis[11]. Ascorbic acid treated model showed intense staining of p63 compared to control (LSE CTL) model (Fig. 2). p63 positive cells were usually observed at basal/suprabasal area but numerous cells at mid-epidermis were also stained with anti-p63 antibody in ascorbic acid treated model. α_6 integrin, known as matrix receptor, was expressed in basement membrane zone in normal skin[12]. In LSE CTL model, focal staining of α_6 integrin was observed along the dermo-epidermal junction, but linear staining of α_6 integrin was observed in LSE Vit. C model (Fig. 3).

Ascorbic acid promotes normal human keratinocyte proliferation

Keratinocytes were treated with various concentrations of ascorbic acid ranging from 0-100 μg/mL for 72 h, and cell viability was determined by using the MTT assay. A dose-dependent increase in cell viability was observed with increasing concentrations of ascorbic acid (Fig. 4).

Ascorbic acid induces activation of EGF-R and phosphorylation of ERK

Because ascorbic acid induced proliferation of

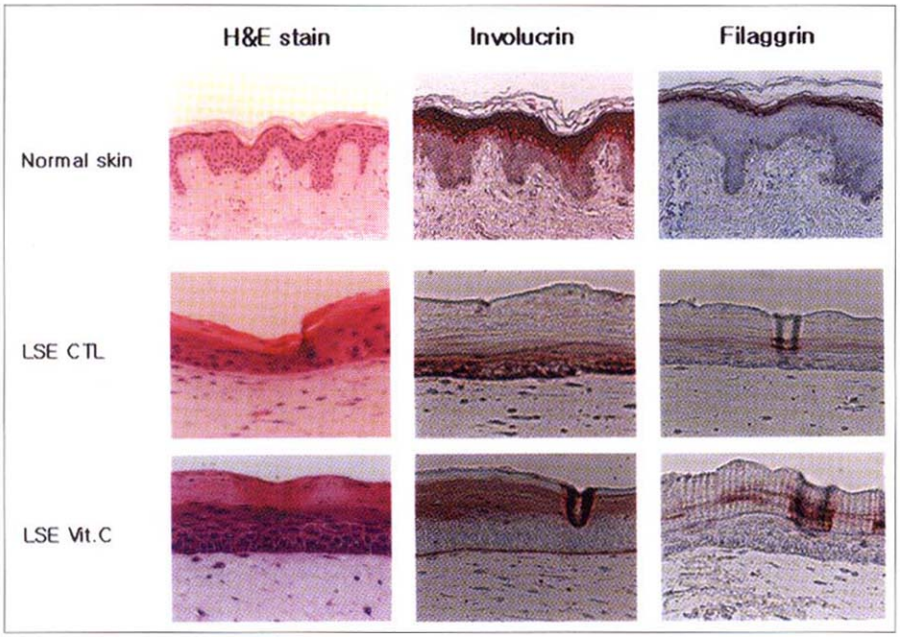

Fig. 1. Immunohistochemical staining of involucrin and filaggrin in control and ascorbic acid treated LSE model (× 200).

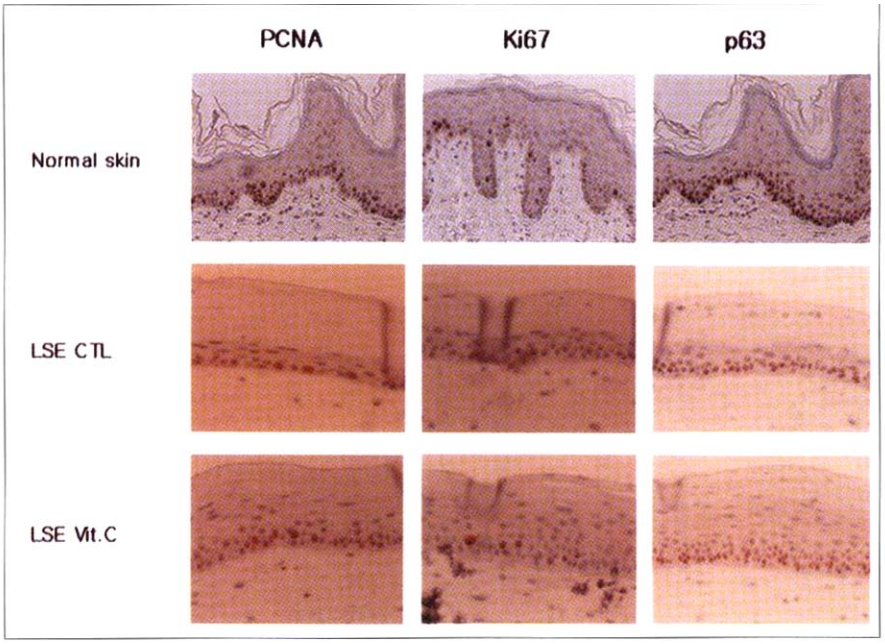

Fig. 2. Immunohistochemical staining of PCNA, Ki67, and p63 in control and ascorbic acid treated LSE model (× 200).

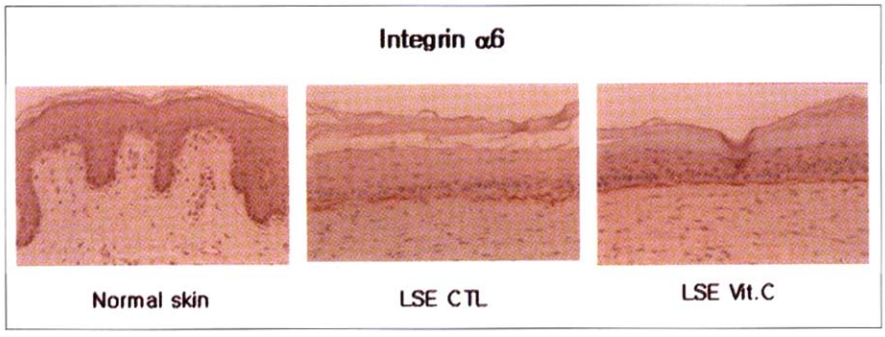

Fig. 3. Immunohistochemical staining of integrin alpha 6 in control and ascorbic acid treated LSE model (× 200).

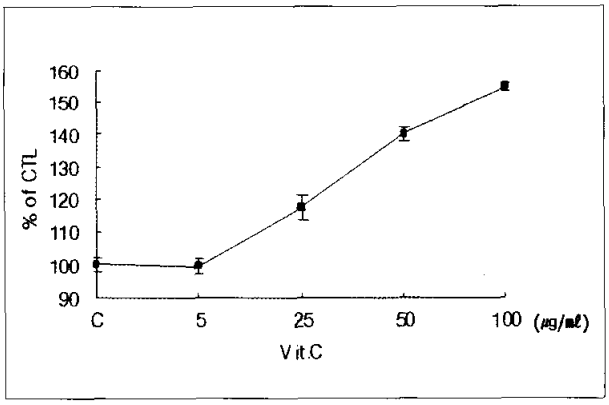

Fig. 4. Effects of ascorbic acid on the proliferation of keratinocytes. Cells were cultured with ascorbic acid for 3 days, and cell viability was assessed by MTT assay. Results are the average of three independent experiments.

keratinocytes, it was tested whether ascorbic acid could induce activation of EGF-R, which is essential for proliferation of keratinocytes. Interestingly, ascorbic acid induced elevation and phosphorylation of EGF-R at a dose-dependent manner 72 h after ascorbic acid treatment. Furthermore, phosphorylation of ERK was also elevated 72 h after ascorbic acid treatment in a dose-dependent manner (Fig. 5). In the literature, it has been reported that ERK phosphorylation triggered transactivation of EGF-R[13]. Therefore, we studied the effect of ascorbic acid on immediate ERK phosphorylation. Results showed that ascorbic acid also induced immediate phosphorylation of ERK after ascorbic acid treatment. We confirmed the results with independently performed experiments (Fig. 6). When the kinetics of EGF-R and ERK phosphorylation were compared, it is suggested that immediate and sustained phos-

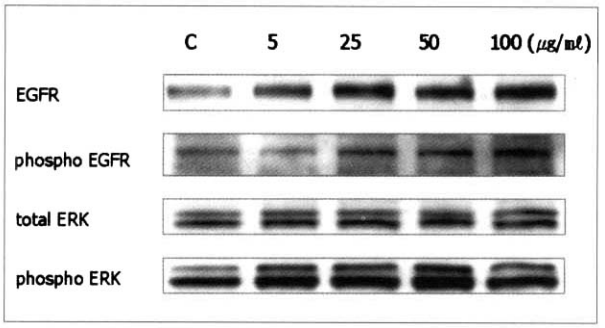

Fig. 5. Delayed effects of ascorbic acid on EGF-R, total ERK, phosphorylation of EGR-R and ERK. Seventy two hours after ascorbic acid treatment, whole cell lysates were prepared and subjected to western blot analysis with antibodies against EGF-R, phospho-specific EGF-R, phosphorylation independent ERK, and phospho-specific ERK. C, negative control.

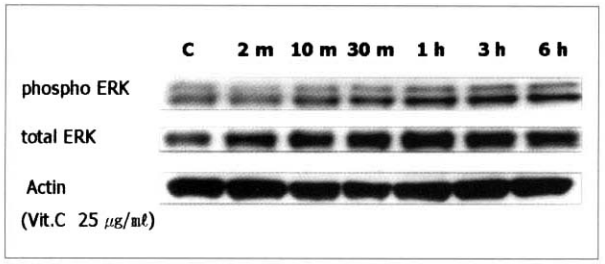

Fig. 6. Immediate effects of ascorbic acid on total ERK and ERK phosphorylation. As shown in figure, whole cell lysates were prepared and subjected to western blot analysis with antibodies against phosphorylation independent ERK and phospho-specific ERK. C, negative control.

phorylation of ERK may increase the expression of EGF-R at 72h after ascorbic acid treatment.

DISCUSSION

Skin equivalent model provides a new investigating system to study the role of extracellular matrix and dermal components such as collagen, basement membrane components, and fibroblasts, which contribute to cell-cell and cell-matrix interactions[6,14,15]. Ascorbic acid is known to increase the biosynthesis of collagen, and the incubation of LSE with ascorbic acid extends cellular viability, promotes formation of epidermal barrier *in vitro*, and promotes engraftment[1,2]. We also observed that ascorbic acid could affect the epidermalization in reconstructed human epidermis. However, molecular mechanism of these findings is not clarified yet. In our study, LSE was reconstructed and characteristic multilayering and stratification of epidermis was observed. Incubation of LSE in ascorbic acid-containing medium facilitates the formation of LSE with thick epidermis, more compact horny layer, and more regular arrangement of basal cells. These findings suggest that ascorbic acid regulates the proliferation and differentiation of keratinocytes. In literature, it is reported that ascorbic acid affects differentiation of keratinocytes[16]. In our experiment, we observed well developed horny layer and these findings are consistent with previous reports. However, it seemed that ascorbic acid could regulate proliferation of keratinocytes when considering thick epidermis of ascorbic acid treated LSE. These findings were also supported by immunohistochemical staining. When LSE was immunostained with antibody against involucrin and filaggrin, similar staining patterns were observed in both models, except negative staining of involucrin in lower epidermis of ascorbic acid treated model. Histological findings such as thick epidermis and negative involucrin staining of lower epidermis suggest that ascorbic acid is important for prevention of premature differentiation of keratinocyte. In other words, ascorbic acid is necessary for the maintenance of proliferative potential of keratinocytes in the reconstructed skin equivalent.

PCNA is known to be present through out the cell cycle in proliferating cells[9]. In the skin, PCNA expression is known to be present mainly in the basal layer of normal skin. In both models, PCNA positive cells were scattered along the basement membranes and there was no significant differences. Ki67, a nuclear protein that is expressed in proliferating cells, was observed in the basal layer of epidermis.[10] There are also no significant differences in Ki67 expression in these models. The p63 transcription factor belongs to a family that includes two structurally related proteins, p53 and p73[17]. It is reported that p63 is the first gene product definitely distinguishing stem cells from their transient amplifying progeny in stratified squamous epithelia.[11] It was also known that p63(-/-) mice lack stratified epithelia and contain clusters of terminally differentiated keratinocytes on the exposed dermis.[18] p63 expression

is known to be present mainly in the basal/suprabasal layer of epidermis. Interestingly, intense staining of p63 was observed in ascorbic acid treated models. In addition, p63 positive cells were observed even in mid-epidermis of reconstructed skin equivalent. It is not clear why the expression of p63 is significantly elevated in ascorbic acid treated LSE model and most of p63 positive cells are not stained with antibody against PCNA. These findings need to be clarified in future. However, numerous cells in LSE Vit. C model were stained with antibody for p63 and these findings suggest that ascorbic acid have an important role in the regulation of proliferative potential of human keratinocytes.

Furthermore, we studied the effect of ascorbic acid on normal human keratinocytes proliferation and the molecular mechanism of proliferative effects. Interestingly, ascorbic acid stimulates the proliferation of keratinocyte at a dose dependent manner. Because ascorbic acid has proliferative effect on keratinocytes, we investigated whether these effects are related to the regulation of ERK and EGF-R. Results showed that ascorbic acid induced both immediate and delayed activation of ERK in cultured normal human keratinocytes. Western blotting also showed that ascorbic acid increased the level of EGF-R at a dose-dependent manner in cultured normal human keratincoytes. These findings suggest that ascorbic acid induces proliferation through up-regulation of EGF-R in normal human keratinocytes. Recently it has been reported that ERK phosphorylation triggered transactivation of EGF-R[13]. Our observation suggests that ascorbic acid could induce the proliferation of keratinocytes through ERK mediated EGF-R expression. Finally, effect on integrin expression was investigated. α_6 integrin, a detection marker of extracellular adhesion receptors, is distributed along the dermal epidermal junction and this means the existence of hemidesmosomes[12,19,20]. Our results showed that ascorbic acid is very important for the regulation of integrin expression.

Thus it can be said that skin equivalent model, which was reconstructed with ascorbic acid enriched medium, seems to be a good substitute of human skin.

Acknowledgement

This research was supported by a grant (SC13111) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

REFERENCES

1. Steven TB, Andrew PS, Viki BS, Glenn DW: Vitamin C Regulates Keratinocyte Viability, Epidermal Barrier, and Basement Membrane *In Vitro*, and Reduces Wound Contraction After Grafting of Cultured Skin Substitutes. J Invest Dermatol 2002;118(4):565-572.
2. Bell E, Rosenberg M, Kemp P, Gay R, Green GD, Muthukumarar NC: Recipes for reconstituting skin. J Biomech Eng 1991;113(2):113-119.
3. Regnier M, Darmon M: Human epidermis reconstructed *in vitro*: a model to study keratinocyte differentiation and its modulation by retinoic acid. *In Vitro* Cell Dev Biol 1989;25:1000-1008.
4. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975;6:331-433.
5. Bell E, Ivarsson B, Merrill C: Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. Proc Natl Acad Sci U S A 1979;76:1274-1278.
6. Auger FA, Rouabhia M, Goulet F, Berthod F, Moulin V, Germain L: Tissue-engineered human skin substitutes developed from collagen-populated hydrated gels: clinical and fundamental applications. Med Biol Eng Comput 1998;36:801-812.
7. Li ER, Owens DM, Djian P, Watt FM: Expression of involucrin in normal, hyperproliferative and neoplastic mouse keratinocytes. Exp Dermatol 2000;9(6):431-438.
8. Ishida YA, Takahashi H, Presland RB, Dale BA, Iizuka H: Translocation of profilaggrin N-terminal domain into keratinocyte nuclei with fragmented DNA in normal human skin and loricrin keratoderma. Lab Invest 1998;78(10):1245-1253.
9. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H: Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. Nature 1987;326:515-517.
10. Rijzewijk JJ, Van Erp PE, Bauer FW: Two binding sites for Ki67 to quiescent and cycling cells in human epidermis. Acta Derm venereal 1989;69: 512-515.
11. Pellegrini G, Dellambra E, Golisano O, et al.: p63

identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 2001;98:3156-3161.
12. Hertle MD, Adams JC, Watt FM: Integrin expression during human epidermal development *in vivo* and *in vitro*. Development 1991;112:193-206.
13. Marques SA, Dy LC, Southall MD, et al.: The platelet-activating factor receptor activates the extracellular signal-regulated kinase mitogen-activated protein kinase and induces proliferation of epidermal cells through an epidermal growth factor-receptor-dependent pathway. J Pharmacol Exp Ther 2002;300(3):1026-1035.
14. Bell E, Ehrlich HP, Buttle DJ, Nakatsuji T: Living tissue formed *in vitro* and accepted as skin-equivalent tissue of full thickness. Science 1981;211: 1052-1054.
15. Boyce ST, Hansbrough JF: Biologic attachment, growth, and differentiation of cultured human epidermal keratinocytes on a graftable collagen and chondroitin-6-sulfate substrate. Surgery 1988;103: 421-431.
16. Savini I, Catani MV, Rossi A, Duranti G, Melino G, Avigliano L: Characterization of keratinocyte differentiation induced by ascorbic acid: protein kinase C involvement and vitamin C homeostasis. J Invest Dermatol 2002;118(2):372-379.
17. Lohrum MA, Vousden KH: Regulation and function of the p53-related proteins: same family, different rules. Trends Cell Biol 2000;10(5):197-202.
18. Annie Y, Ronen S, Deqin S, et al.: p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 1999;398 (6729):714-718.
19. Moll R, Moll I: Epidermal adhesion molecules and basement membrane components as target structures of autoimmunity. Virchows Arch 1998;432: 487-504.
20. Sonnenberg A, Calafat J, Janssen H, et al.: Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. J Cell Biol 1991; 113:907-917.