Lyophilized Keratinocyte Cell Lysates Contain Multiple Mitogenic Activities and Stimulate Closure of Meshed Skin Autograft–Covered Burn Wounds with Efficiency Similar to That of Fresh Allogeneic Keratinocyte Cultures

Luc Duinslaeger, M.D., Gilbert Verbeken, Pascal Reper, M.D., Bernard Delaey, Ph.D., Stefaan Vanhalle, and Alain Vanderkelen, M.D.

Brussels, Belgium

For several years, grafting with allogeneic keratinocyte cultures has been used successfully as a wound-healing therapy both by us and by many other groups. Since their postgrafting survival time is limited, the effect of these cultures is generally explained by the production of wound repair–stimulating factors that promote proliferation and migration of resident cells. In this study we show that lysates of cultured keratinocytes contain mitogenic activity for keratinocytes, endothelial cells, and fibroblasts. In addition, the lysates inhibit the contraction of collagen gels by human skin fibroblasts. On the basis of these observations and of in vivo data obtained by ourselves and others, we have evaluated the effect of total keratinocyte lysates on the healing of meshed skin autograft–covered burn wounds. Twenty burn wounds were tangentially excised and autografted with one to three meshed conventional skin transplants. An area treated with a gel containing lysated keratinocyte cultures was compared with an area treated with placebo-gel in terms of epithelialization on day 5. In six patients an additional fresh keratinocyte alloculture was applied as a positive control.

Results indicate that the newly formed epithelium (difference between percentage of epithelialization on day 5 and on day 0) was 31.1 percent in the treated area compared with 16.5 percent in the placebo area. This result is comparable with the value obtained by treatment with fresh keratinocyte allocultures, namely, 33.8 percent. These figures show a twofold stimulation of epithelialization. (*Plast. Reconstr. Surg.* 98: 110, 1996.)

Since the introduction of efficient keratinocyte culturing techniques,[31] cultured epithelium autografting has become a widely established tool in the armamentarium of the burn surgeon. However, the time involved in expanding autologous keratinocyte cultures to obtain a surface area sufficiently large to allow the grafting of extensive burns is a serious drawback in many cases. Therefore, several centers have explored the possibility of using cultured allogeneic keratinocytes for the treatment of burns, donor sites, and chronic ulcers.[1–5] Our own studies also have demonstrated the effectiveness of cultured allogeneic keratinocyte sheets for enhancing the closure of split-thickness meshed skin autografts in burn patients. Nevertheless, even the use of cultured allogeneic epithelium is still tempered by several drawbacks, especially with respect to cost and availability of donor skin, technical infrastructure, and know-how. In addition, several studies have shown that the survival time of these allocultured grafts is limited and that they are gradually replaced by autologous cells.[6–8] Therefore, the observed enhancement of epithelial coverage by these grafts is not due to permanent "take" of the grafts but generally has been attributed to an indirect stimulation of the repair mechanisms of the recipient. This may be mediated, for instance, by the production of growth factors, which may induce wound closure by stimulating the migration and proliferation of resident epithelial cells present in the wound bed. These resident keratinocytes may be derived from surviving epidermal appendages (e.g., in second-degree burns), from the

From the Burn Center of Brussels and Innogenetics, Ghent. Received for publication August 9, 1994; revised July 5, 1995.

wound edges, or from split-thickness skin autografts. In this study we have substantiated this theory by showing that keratinocyte cell extracts are mitogenic toward multiple cell types and that whole lysates of these cells are able to significantly enhance the closure of split-thickness skin autografts. We have generically named these wound repair–stimulating factors produced by keratinocytes *keratinocyte-stimulating factors.*

PATIENTS AND METHODS

Patient Population and Study Design

A total of 20 lyophilizate applications (labeled *A* to *T*) were performed at the Military Hospital Burn Center on 17 patients (12 males and 5 females) after obtaining informed consent. The age of the patients ranged from 7 to 63 years, with a mean of 36 years. The total body surface area burned ranged from 3 to 49 percent, with a mean of 23 percent. The patients presented with both deep second- and third-degree burn wounds. Exclusion criteria for this clinical study were postoperative bleeding, infection of the wound, unwrapping of wounds at other timepoints than 5 days postoperatively, and sliding of the application template.

Of each wound, a 25-cm^2 area was treated with keratinocyte lysate and an adjacent area with a control gel (see *Clinical Application Protocol*). Selection of control and lysate-treated areas was performed at random. Application of lysate or control and evaluation were performed double-blind.

The purpose of the study was to compare the amount of newly formed epithelium in both conditions 5 days after surgery (which was found to be the optimal time for evaluation). Therefore, wound closure on day 5 was quantitatively determined (see *Method of Evaluation*). The actual percentage of newly formed epithelium after 5 days was calculated by subtracting the average original surface area (i.e., the day 0 closure value) of a meshed autograft from the total closed area on day 5. This average day 0 closure value was determined on a population of 26 of the wounds.

Only from 9 of the 20 wounds in the study population (wounds *C, K, L, M, N, P, Q, R,* and *T*) did we have the actual day 0 closure values from both the lysate and the control areas. Therefore, a separate analysis was performed on this subpopulation.

In 6 cases within the population (applications *E, F, H, L, Q,* and *S*), wounds were treated additionally with fresh keratinocyte cultures and quantitatively evaluated. This subpopulation was used to compare the effect of the lysate with that produced by fresh cultures.

In Vitro Culture of Keratinocytes

Keratinocyte cultures were established from skin specimens obtained from healthy donors with ages ranging between 15 and 59 years (mean 36 years) who tested negative for human immunodeficiency virus (HIV), hepatitis B virus, hepatitis C virus, and syphilis. All donors met the criteria for organ donation. Keratinocytes were cultured in mitomycin C–treated feeder layers, as described by Rheinwald and Green.[31] The primary keratinocytes were used for grafting upon reaching confluency in passage 2. Twenty-four hours before picking up, the cells were rinsed three times with physiologic saline and put in a culture medium without fetal calf serum or additives.

Preparation of Keratinocyte Lysates and Cell Extracts

To prepare the lysates, we used seventy-seven 175-cm^2 keratinocyte cultures obtained from three donors (30 cultures from the first, 30 cultures from the second, and 17 cultures from the third donor) that were stored at −70°C as cell pellets. Before preparing the lyophilizate, an additional microbiologic contamination test was performed. After thawing, the cells were subjected to a hypotonic shock by addition of ultrapure water to a final volume of 3 ml per culture. The tubes were vortexed until a homogeneous suspension was obtained, and the resulting cell lysates were passed three to four times through a syringe. These lysates were subsequently lyophilized in order to allow application in a smaller volume. Lyophilization was carried out in a sterile way in glass vials, each containing 1.7 ml of keratinocyte lysate. This amount, corresponding to a keratinocyte culture of 100 cm^2, was used for the treatment of a single application zone of 25 cm^2, resulting in a fourfold "concentrated" form of lyophilizate as compared with fresh cultures. Immediately before use, the lyophilizate was rehydrated in a suitable volume of 4% isotonic hydroxyethylcellulose gel (Idroramnosan, Federa, Belgium).

For preparation of cell extracts, cultures were sonicated (10 cycles of 10 seconds each) in PBS (20 ml/175 cm^2 of cells), followed by centrifugation at 48,000*g* for 45 minutes. The protein

concentration in these extracts was 600 μg/ml on average.

Preparation of Keratinocyte Cultures for Grafting

Keratinocyte cultures were detached from the culturing flask by treatment with 20 ml dispase (2.5 μg/ml) for approximately 15 minutes. A carrier gauze (N-terface, Hospitera, Belgium) was put on top of the keratinocytes for additional support. The culture could then be taken out of the tissue flask for application onto the wounds (with the basal side of the culture sheet making contact with the wound surface).

Clinical Application Protocol

A large, deep second- or third-degree burn wound was prepared by *subdermal* tangential excision and autografted with one to three meshed split-thickness skin grafts with maximum expansion. Control gel (4% hydroxyethylcellulose gel) and lysate (tantamount to 100 cm^2 of keratinocytes suspended in 4% hydroxyethylcellulose gel) were applied into 5 × 5 cm application zones cut from a 5-mm-thick silicone template (Comesa, Belgium) that was placed on top of the autografted wound. This facilitated application of the gels and ensured their confinement to the application zone. Selection of application sites was performed at random, and application was done in a blinded fashion. To exclude bias due to differential severity of the wounds, care was taken to ensure that excision depth was identical over the whole wound area. The silicone template remained in place for 5 days following surgery and was found to produce no negative effects on the healing rate of the wounds.

In six wounds, the remainder of the burn was treated with fresh allogeneic keratinocyte cultures and served as a positive control.

Method of Evaluation

Five days postoperatively, wounds were unwrapped and photographed in a standardized way. The percentage of closure of the wounds was determined by placing a grid pattern composed of short line segments[29] onto the photographs of the different application zones. The amount of closure was calculated by counting the number of line endpoints falling within closed zones and expressing this as the percentage of the total number of endpoints falling within the application zone. Each photograph was evaluated by three independent, experienced evaluators.

Statistics

The data were analyzed by multiple-factor repeated-measurements ANOVA. The treatment, the rater, and the moments of measurement are within factors. Shapiro-Wilk *W* normality tests for each variable showed no departure of the test populations from normality.

Mitogenic Assays

Mitogenic activity for keratinocytes was assayed by tritium thymidine incorporation on *Balb/MK* keratinocytes as described by Cook et al.[12] Mitogenic assays on fetal bovine heart endothelial cells and on Swiss 3T3 fibroblasts were as described by Van Zoelen.[27]

Results

In Vitro Results

Mitogenic and Contraction-Inhibiting Activity. In order to demonstrate the presence of keratinocyte-stimulating factors in epidermal cell cultures, human keratinocyte extracts were prepared by sonication and centrifugation at 48,000*g* or 100,000*g*. These extracts were shown to contain mitogenic activity for murine *Balb/MK* keratinocytes at dilutions up to 1:36 (Fig. 1, *a*). By comparison with EGF and aFGF standards included in the same assay, fourfold diluted keratinocyte cell extracts were shown to stimulate the cells about as much as 2.5 ng/ml EGF or aFGF. In addition to keratinocytes, the extracts are also mitogenic for other cell types involved in the wound repair process. As shown in Figure 1 (b), a 16-fold diluted extract stimulated the proliferation of murine Swiss 3T3 fibroblasts more potently than 2.5 ng/ml of bFGF. Furthermore, fetal bovine heart endothelial cells also were significantly stimulated by a 20-fold concentrated keratinocyte 100,000*g* cell extract (see Fig. 1, *c*). Finally, apart from containing mitogenic activity toward several cell types, keratinocyte extracts also were shown to inhibit the contraction of collagen lattices by human skin fibroblasts (not shown). This in vitro assay[29] is generally considered as being reminiscent of the contraction process associated with healing of full-thickness skin wounds, a phenomenon ususally considered undesirable during human wound healing.[30] All these in vitro results suggest that keratinocyte cell preparations may positively influence many of the repair mechanisms involved in the wound-healing process.

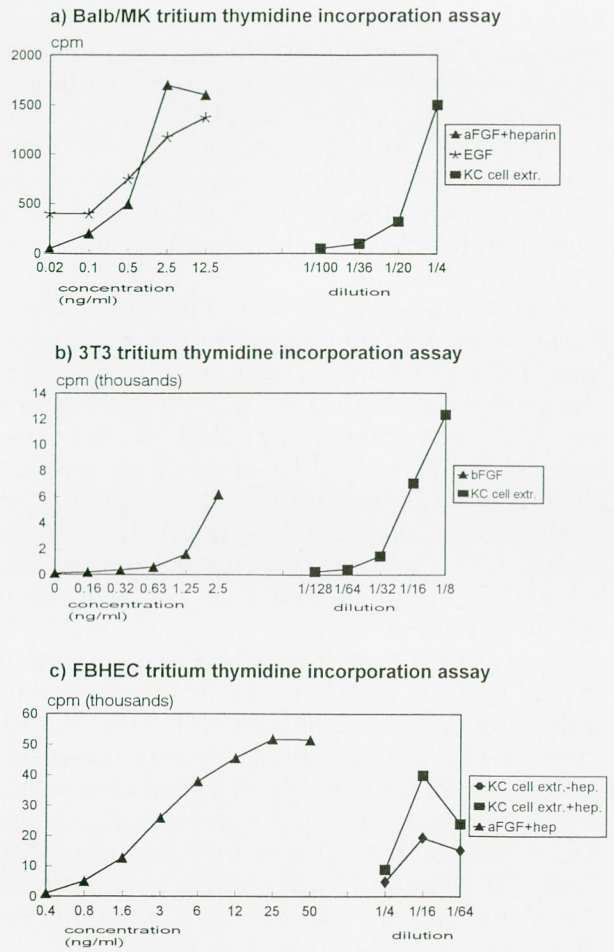

FIG. 1. Mitogenic activity of keratinocyte cell extracts and control growth factors on different cell types. (a) Murine *Balb/MK* keratinocytes stimulated with keratinocyte cell extract (KC cell extr.), epidermal growth factor (EGF), and acidic fibroblast growth factor (aFGF) in the presence of 10 μg/ml heparin. (b) Murine Swiss 3T3 fibroblasts stimulated with keratinocyte cell extract (KC cell extr.) and basic fibroblast growth factor (bFGF). (c) Fetal bovine heart endothelial cells (FBHEC) stimulated with a 100,000*g* keratinocyte cell extract (KC cell extr., prepared in a 20-fold concentrated form) in the presence or absence of 10 μg/ml heparin and with acidic fibroblast growth factor (aFGF) in the presence of 10 μg/ml heparin.

Stability. The keratinocyte stimulatory activity of the extracts was found to be remarkably stable, since most of the *Balb/MK* mitogenic activity was preserved after a 2-day incubation, even at 37°C. After prolonged incubation, the formation of a precipitate was noted, however, presumably due to reaggregation of membrane vesicles. Upon resolubilization in detergent, these precipitates were found to contain significant amounts of keratinocyte-stimulating activity. Therefore, we decided to use a total keratinocyte lysate in the clinical study instead of using only the soluble cell extract fraction.

Clinical Results

In this study, keratinocyte lysates were prepared by hypotonic shock and lyophilization. The lyophilizate was reformulated in a neutral carrier vehicle (Idroramnosan gel) and applied on top of subdermally excised burn wounds that had been covered with one to three meshed skin autografts. Each 25-cm^2 application area received an amount of lysate derived from 100 cm^2 of keratinocyte culture. Immediately adjacent to the lysate application area, a control area was treated with vehicle only. A total of 20 wounds were treated in 17 patients. Five days after treatment, wounds were unwrapped, and the amount of wound closure was determined and expressed as percentage of total wound area. This shows an average closure of 78.73 percent (SD 12.98 percent) and 64.05 percent (SD 14.4 percent) in lysate- and placebo-treated wounds, respectively. Statistical analysis by ANOVA showed the difference in closure between the placebo- and lysate-treated groups to be statistically significant ($p < 0.00006$) and independent of the evaluator ($p < 0.24$). This is also illustrated in Figure 3, which shows a representative meshed autograft–covered third-degree burn treated with keratinocyte lysate and control gel during and 5 days after surgery. To calculate the amount of newly formed epithelium, it has to be taken into account that part of the wound is already closed

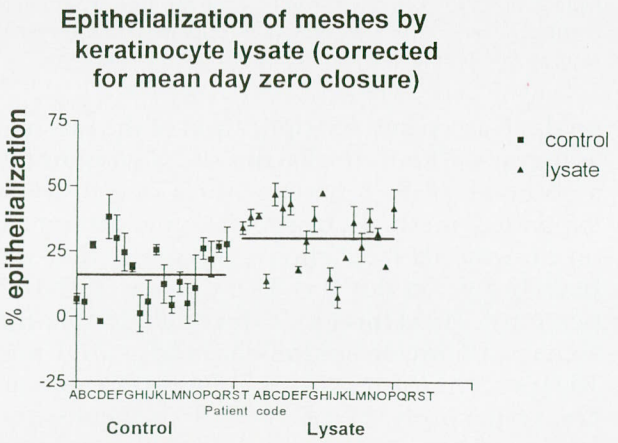

FIG. 2. Plot of percentage of epithelialization in wounds treated with keratinocyte lysate or control gel (measured 5 days after surgery). Epithelialization values were obtained by subtracting the average day 0 closure (47.6 percent; see text) from the measured day 5 closure values. Patients are labeled *A* to *T*. Error bars represent standard error of three measurements, performed independently by three different evaluators. Average epithelialization percentage for both treatment groups (16.5 and 31.1 percent, respectively) is indicated by the thick horizontal lines.

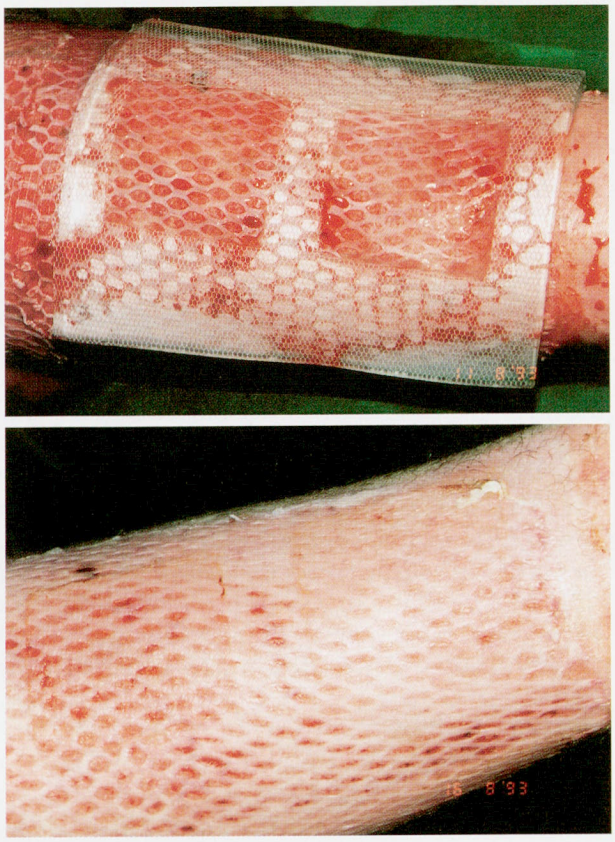

FIG. 3. Third-degree burn wound, tangentially excised and grafted with a meshed split-thickness autograft. (*Above*) Immediately after surgery. A placebo hydroxyethylcellulose gel and a keratinocyte lysate–containing gel are applied in the left and right windows of the silicone application template, respectively. In the zone left of the silicone template, a fresh keratinocyte alloculture was applied as a positive control. (*Below*) Five days after surgery. Note the almost complete reepithelialization of the right (keratinocyte lysate–treated) zone as compared with the left (placebo gel) zone.

on day 0 as a result of application of the meshed autografts. Theoretically, this should amount to a coverage of 33.3 percent for a one to three expanded mesh. Retrospective measurements on 26 wounds have shown, however, that the real closure on day 0 is 47.6 percent (SD 10.5 percent). Therefore, we can conclude that the actual amount of epithelialization is 31.1 and 16.5 percent for lysate- and placebo-treated areas, respectively (see Fig. 2). This means that lysate treatment results in an almost twofold increase in epithelialization within 5 days.

The possibility exists that divergence from the average 47.6 percent of closure on day 0 would have biased the results in some wounds. To exclude this, we recalculated the epithelialization values of 9 wounds, taking into account the actual percentage of closure on day 0 for these wounds (for the other 11 wounds, insufficient photographic records were available to allow calculation of day 0 values). This results in very similar figures, with an average epithelialization of 30.43 percent (SD 14.36 percent) and 19.5 percent (SD 11.53 percent) for lysate- and placebo-treated wounds, respectively. Again, ANOVA shows the difference between both treatment groups to be highly significant ($p < 0.0035$).

Next, we questioned whether the effect of the lysate would be comparable with that of fresh keratinocyte cultures. This was done by evaluating day 0 and day 5 closure levels in 6 wounds that had been treated with fresh keratinocyte allocultures as well. This showed an average closure of 78.28 percent (SD 12.72 percent) in fresh culture–treated areas, a figure that was not significantly different ($p < 0.89$) from closure values in the lysate-treated areas (77.09 percent, SD 14.34 percent).

Our results thus demonstrate that treatment of meshed autograft–covered burn wounds with lyophilized keratinocyte lysates significantly enhances the healing of the meshed-graft interstices. Moreover, the keratinocyte lysate treatment results in wound-healing effects similar to those of living allocultured keratinocytes. Together with our in vitro results, this confirms the viewpoint that allocultured keratinocyte sheets enhance wound healing mainly through the stimulation of resident cells and warrant further investigations on the mechanisms and factors mediating this effect.

DISCUSSION

The wound repair–stimulating effects of keratinocyte allograft cultures generally have been explained in terms of the production of molecular mediators by these cells. Indeed, numerous growth factors and cytokines have been reported to be synthesized by epidermal cells. These include TGF-α, aFGF, bFGF, PDGF, TGF-β, amphiregulin, HB-EGF, NGF, TNF-α, IL-1α, IL-1β, IL-6, IL-8, GM-CSF, G-CSF, and M-CSF.[9–15] Many of these factors have important effects on one or more stages of the wound-healing process. Therefore, although they may survive only temporarily on the wound bed, keratinocyte allocultures may nevertheless actively promote the wound-healing process through the release of the various factors mentioned above and by providing an effective wound cover. Eisinger et al.[16] have shown that keratinocyte extracts and culture supernatants indeed contain factors that promote keratinocyte

proliferation and inhibit fibroblasts. Moreover, application of these so-called epidermal cell–derived factors to surgical wounds in pigs profoundly stimulated reepithelialization. In this study also, we have shown that keratinocyte cell extracts contain mitogenic activity toward various cell types implicated in the repair mechanism: keratinocytes, endothelial cells, and fibroblasts. We have collectively called these activities *keratinocyte-stimulating factors* (KSFs). Comparison of the stimulation values obtained with the keratinocyte cell extract with those obtained with known growth factors shows that a fourfold diluted extract stimulates the target cells to the same extent as 2.5 ng/ml EGF or aFGF. In interpreting this, it has to be taken into account that the exact nature and specific activity of the individual keratinocyte-stimulating factors are still unknown. Moreover, the observed activity is the net result of multiple factors that may interact with each other in a synergistic or antagonistic way. Apart from being mitogenic, the extracts also were found to inhibit collagen contraction by human skin fibroblasts in vitro. This inhibition of contraction in vitro, which also was reported by Eisinger et al.,[16] may reflect the improved cosmetic appearance of wounds treated with allocultured keratinocyte sheets in combination with meshed skin autografts frequently observed in our hands. The finding of so many different activities in a cell extract is not entirely surprising, in view of the multitude of growth factors known to occur entirely or partially in a cell-associated form. This is the case for aFGF, bFGF, TGF-α, amphiregulin, and HB-EGF. Moreover, some of these factors are produced as precursor forms that are physically linked to the cell surface, possibly under a biologically active form.[17–21] Our finding that insoluble components in the cell extract, presumably of membranous nature, also contain significant keratinocyte mitogenic activity also points in this direction. In terms of stability, we have shown that mitogenic activity within the cell extracts is maintained over prolonged time periods, even at 37°C and in the absence of protease inhibitors. Although this may seem surprising at first, it has been shown that some growth factors, such as the FGFs, are highly stabilized upon association with heparin or similar extracellular matrix–derived polyanions.[22–25] In addition, limited proteolysis by specific proteases may result in the release of soluble growth factors from membrane-bound precursors,[17,26] an effect that may temporarily counterbalance proteolytic breakdown. In view of these observations, we feel that the use of a keratinocyte cell extract, or even a complete cell lysate, for promoting wound repair is a sensible approach.

Treatment of severe burn wounds with a combination of meshed skin grafts and allocultured keratinocyte sheets is a technique that has been used successfully for many years in our center. On the basis of in vitro and in vivo data obtained by ourselves and others, we have now extended this technology one step further by using total keratinocyte lysates instead of living cells. As indicated by our data, 5 days after grafting, keratinocyte lysates produce closure values of the meshed autograft interstices similar to those of fresh cultures. In comparison with control areas treated with meshed skin autografts only, the lysates produce a newly formed epithelial coverage that is 50 to 90 percent higher. In all cases, the observed effects are statistically highly significant. In evaluating these results, it has to be taken into account that in this study no attempt was made to optimize the lysate treatment. Indeed, it may be expected that the observed healing could still be improved by adaptation of the dose, number of treatments, way of delivery, inclusion of stabilizers, etc. The amount of mitogenic activity delivered to the wounds in this study is roughly equivalent to that obtained with 30 to 60 ng of epidermal growth factor, as estimated from bioassay data on keratinocyte extracts. This is more within the physiologic concentration range for growth factors, in contrast with the supraphysiologic concentrations of recombinant growth factors used in most in vivo studies, where microgram amounts are usually administered. The fact that we were able to obtain significant stimulation of epithelialization with these comparatively low amounts of mitogenic activity may have different explanations. First, it appears that the growth factor activity in the cell lysates is present in a highly stabilized form, ensuring a prolonged effect even with a single application. Next, there is increasing evidence that combinations of factors, such as present in the total lysates, may be much more clinically efficient than application of a single factor. Also, it is possible that the keratinocyte-stimulating factor activity detected by in vitro assays on crude cell extracts is underestimated, because the preparations also may contain inhibitory factors or because some factors act antagonistically in these assays. Finally, it cannot be excluded that in addition to

growth factors, keratinocyte lysates contain other wound repair–stimulating components. These may include extracellular matrix molecules that may promote keratinocyte adhesion and migration, proteases that may help in debridement of necrotic tissue and liberation of cell-associated soluble growth factors, and nutritional components that may provide a favorable microenvironment for cell proliferation by satisfying metabolic needs.

In conclusion, the technology presented in this paper may represent a further extension of the keratinocyte alloculture grafting technique, bringing it within reach of those centers which do not have the expertise or logistics to set up their own culturing facilities. In addition, it confirms some of the current views on the biologic mechanisms by which allocultured keratinocytes, despite their short postgrafting survival time, are able to promote skin wound healing.

Dr. Luc Duinslaeger
Burn Center Brussels
Bruynstraat, 1
1120 Neder-Over-Heembeek, Belgium

ACKNOWLEDGMENTS

The work described in this paper was supported by grant VLAB/072 from the Flemish Biotechnology Action Program (VLAB).

We also wish to thank Kathy Acke, Peter De Corte, and Marleen De Fruyt for expert technical assistance and STATAP (Ghent, Belgium) for statistical analysis of the clinical data.

REFERENCES

1. Madden, M. R., Finkelstein, J. L., Staiano-Coico, L., et al. Grafting of cultured allogeneic epidermis on second- and third-degree burn wounds on 26 patients. *J. Trauma* 26: 955, 1986.
2. Achauer, B. M., Hewitt, C. W., Black, K. S., et al. Long-term skin allograft survival after short-term cyclosporine treatment in a patient with massive burns. *Lancet* 1: 14, 1986.
3. De Luca, M., Albanese, E., Bondanza, S., et al. Multicenter experience in the treatment of burns with autologous and allogenic cultured epithelium, fresh or preserved in a frozen state. *Burns* 15: 303, 1989.
4. Fratianne, R., Papay, F., Housini, I., et al. Keratinocyte allografts accelerate healing of split-thickness donor site: Applications for improved treatment of burns. *J. Burn Care Rehabil.* 14: 148, 1993.
5. Teepe, R. G., Roseeuw, D. I., Hermans, J., et al. Randomized trial comparing cryopreserved epidermal allografts with hydrocolloid dressings in healing chronic venous ulcers. *J. Am. Acad. Dermatol.* 29: 982, 1993.
6. Auböck, J., Irschick, E., Romani, N., et al. Rejection, after a slightly prolonged survival time, of Langerhans cell-free allogeneic cultured epidermis used for wound coverage in humans. *Transplantation* 45: 730, 1988.
7. Gielen, V., Faure, M., Mauduit, G., and Thivolet, J. Progressive replacement of human cultured epithelial allografts by recipient cells as evidenced by HLA class I antigens expression. *Dermatologica* 175: 166, 1987.
8. Rouabhia, M., Germain, L., Bélanger, F., and Auger, F. Cultured epithelium allografts: Langerhans cell and Thy-1$^+$ dendritic epidermal cell depletion effects on allograft rejection. *Transplantation* 56: 259, 1993.
9. Luger, T. A., and Scwarz, T. Evidence for an epidermal cytokine network. *J. Invest. Dermatol.* 95(6 Suppl.): 100S, 1990.
10. Ansel, J., Perry, P., Brown, J., et al. Cytokine modulation of keratinocyte cytokines. *J. Invest. Dermatol.* 94(6 Suppl.): 101S, 1990.
11. McKenzie, R. C., and Sauder, D. N. The role of keratinocyte cytokines in inflammation and immunity. *J. Invest. Dermatol.* 95(6 Suppl.): 105S, 1990.
12. Cook, P. W., Mattox, P. A., Keeble, W. W., et al. Heparin sulfate regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. *Mol. Cell. Biol.* 11: 2547, 1991.
13. Dabin, I., and Courtois, Y. Acidic fibroblast growth factor overexpression in corneal epithelial wound healing. *Growth Factors* 5: 129, 1991.
14. Hashimoto, K., Higashiyama, S., Asada, H., et al. HB-EGF, a new member of EGF family, is an autocrine growth factor for normal human keratinocytes. (Abstract) *J. Invest. Dermatol.* 101: 411, 1993.
15. Tron, V. A., Coughlin, M. D., Jang, D. E., et al. Expression and modulation of nerve growth factor in murine keratinocytes (Pam212) *J. Clin. Invest.* 85: 1085, 1990.
16. Eisinger, M., Sadan, S., Silver, I. A., and Flick, R. B. Growth regulation of skin cells by epidermal cell–derived factors: Implications for wound healing. *Proc. Natl. Acad. Sci. U.S.A.* 85: 1937, 1988.
17. Pandiella, A., and Massagué, J. Cleavage of the membrane precursor for transforming growth factor α is a regulated process. *Proc. Natl. Acad. Sci. U.S.A.* 88: 1726, 1991.
18. Brachmann, R., Lindquist, P. B., Nagashima, M., et al. Transmembrane TGF-α precursors activate EGF/TGF-α receptors. *Cell* 56: 691, 1989.
19. Wong, S. T., Winchell, L. F., McCune, B. K., et al. The TGF-α precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell* 56: 495, 1989.
20. Higashiyama, S., Abraham, J. A., Miller, J., et al. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 251: 936, 1991.
21. Plowman, G. D., Green, J. M., McDonald, V. L., et al. The amphiregulin gene encodes a novel epidermal growth factor–related protein with tumor-inhibitory activity. *Mol. Cell Biol.* 10: 1969, 1990.
22. Volkin, D. B., Tsai, P. K., Dabora, J. M., et al. Physical stabilization of acidic fibroblast growth factor by polyanions. *Arch. Biochem. Biophys.* 300: 30, 1993.
23. Gospodarowicz, D., and Cheng, J. Heparin protects basic and acidic FGF from inactivation. *J. Cell Physiol.* 128: 475, 1986.
24. Kajio, T., Kawahara, K., and Kato, K. Stabilization of basic fibroblast growth factor with dextran sulfate. *FEBS Lett.* 306: 243, 1992.
25. Gallagher, J. T., and Turnbull, J. E. Heparan sulfate in the binding and activation of basic fibroblast growth factor. *Glycobiology* 2: 523, 1992.
26. Cappelluti, E., Strom, S. C., and Harris, R. B. Potential

role of two novel elastase-like enzymes in processing pro-transforming growth factor-α. *Biochemistry* 32: 551, 1993.

27. Van Zoelen, E. The use of biological assays for detection of polypeptide growth factors. *Prog. Growth Factor Res.* 2: 131, 1990.
28. Green, H., Kehinde, O., and Thomas, J. Growth of cultured epidermal cells into multiple epithelia suitable for grafting. *Proc. Natl. Acad. Sci. U.S.A.* 76: 5665, 1979.
29. Weibel, E. R., Kistler, G. S., and Scherle, W. F. Practical stereological methods for morphometric cytology. *J. Cell Biol.* 30: 23, 1966.
30. Bell, E., Ivarsson, B., and 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.* 76: 1274, 1979.
31. Rheinwald, J. G., and Green, H. Serial cultivation of strains of epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell* 6: 331, 1975.