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Expression of Interleukin-1α, Interleukin-6, and Basic Fibroblast Growth Factor by Cultured Skin Substitutes before and after Grafting to Full-Thickness Wounds in Athymic Mice

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Objectives: **Cultured skin substitutes (CSSs), consisting of human keratinocytes and human fibroblasts attached to collagen-glycosaminoglycan substrates, have been demonstrated to cover wounds, and may release detectable quantities of growth factors that promote wound healing.**

Materials and Methods: **Basic fibroblast growth factor (bFGF), interleukin-1α (IL-1α), and interleukin-6 (IL-6) were assayed by enzyme linked immunosorbent assay and immunohistochemistry in CSSs in vitro and at days 1, 3, 7, 14, and 21 after grafting to full-thickness wounds in athymic mice.**

Measurements and Main Results: **When isolated cells were tested, IL-1α was found to come primarily from the keratinocytes, whereas bFGF was from the fibroblasts. Combinations of both cell types in the CSSs resulted in a synergistic enhancement of IL-6 expression. Quantities of all three cytokines from CSSs were greater in vitro compared with in vivo levels at all time points after grafting. bFGF increased from day 1 to day 7, and then remained relatively constant until day 21. At day 3 maximal levels of IL-1α were observed. By day 7, IL-1α decreased to approximately 40% of maximal levels, and subsequently increased until day 21. IL-6 levels were highest at day 7 after grafting. All cytokines had reached elevated levels during the time of wound revascularization (days 3–7).**

Conclusions: **The sequence of cytokine synthesis in the wounds (i.e., rapid IL-1α increase followed by IL-6 expression) parallels serum levels reported after a septic challenge. These findings support the hypothesis that the wound is a source of systemic cytokines.**

Key Words: **Basic fibroblast growth factor, Cultured skin substitute, Collagen-glycosaminoglycan, Human keratinocytes, Human fibroblasts, Interleukin-1α, Interleukin-6, Split thickness autografts.**

Wound healing is a complex process involving clotting, inflammation, fibroplasia, neovascularization, wound contraction, and tissue remodeling. Cytokines and inflammatory mediators regulate all of these processes by controlling the growth, differentiation, and metabolism of the cells involved in wound healing. It follows intuitively that skin grafts containing viable cells may produce significant amounts of growth factors. In wounds that require skin grafting, the grafts are believed to promote wound closure by releasing a variety of cytokines.[1] Numerous cytokines have been shown to be involved in cutaneous wound healing. Because vascularization of cultured skin substitutes (CSSs) is delayed,[2] the angiogenic factor, basic fibroblast growth factor (bFGF), was evaluated. bFGF affects most cell types involved in tissue repair,[3,4] and topical application of exogenous bFGF accelerates wound healing in vivo.[5–7] CSSs are also extremely susceptible to microbial contamination, with substantial graft loss if colonization occurs. Therefore, the inflammatory mediators interleukin-1α (IL-1α) and interleukin-6 (IL-6) were also evaluated. IL-1α, which is produced by keratinocytes and many other cells,[8] has been shown to stimulate fibroblast and keratinocyte growth, collagen synthesis by fibroblasts, and chemotaxis of keratinocytes in response to injury.[8] IL-6 may also play a role in wound healing because it is expressed by mesenchyme or epithelium-derived cell lines.[9–11] IL-6 stimulates the migration of epithelial cells into open wounds,[10] and reduced expression of IL-6 is also described in a model of impaired wound healing.[12]

Similar to split thickness autografts (STAGs), CSSs may synthesize and release cytokines, and may modulate expression of cytokines in the wound. CSSs consisting of human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates have been studied as an experimental, adjunctive treatment for extensive burns.[2] In a comparison of clinical results, the percentage of CSS engraftment has not been as great as with STAGs.[2] Several deficiencies with this experimental skin graft have been recognized to include slower revascularization, slower keratinization, greater graft loss from microbial contamination, and greater mechanical fragility compared to STAGs. Improvement in CSS engraftment has been achieved by addressing the above deficiencies. Hypothetically, evaluation of the molecular mechanisms of healing by these experimental skin grafts may allow the causes of these deficiencies to be identified and corrected to promote optimal healing of grafted wounds. The purpose of this experiment was to evaluate the direct expression of bFGF, IL-1α, and IL-6 proteins in CSSs at serial time points after grafting to athymic mice, and in the components of CSSs in vitro. The location of each cytokine in healing epidermis was determined by immunohistochemistry.

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MATERIALS AND METHODS

Biopolymer Preparation

All skin substitutes consisted of cultured human keratinocytes and fibroblasts attached to type I collagen-glycosaminoglycan (C-GAG) biopolymer substrate.[13] The substrates were prepared as previously described[14] with the following experimental changes. Two solubilities of collagen, insoluble and partially soluble, were combined in equal parts to prepare the C-GAG coprecipitate (0.6% w/v). The C-GAG was casted and lyophilized to produce acellular substrates. Crosslinking of substrates with glutaraldehyde was omitted.[14]

Preparation of CSSs

CSSs were prepared as previously described from C-GAG substrates populated with human dermal fibroblasts and epidermal keratinocytes.[15] Briefly, dermal fibroblasts were inoculated into the porous surface of the C-GAG substrate and allowed to incubate for one day, at which time the substrates were inverted and keratinocytes inoculated onto the nonporous, laminated surface. Composite grafts were submerged for one day in Molecular, Cellular, and Developmental Biology 153 medium containing 0.2 mM calcium. On culture day 2, the calcium concentration was adjusted to 0.5 mM, and bovine pituitary extract was replaced with lipid supplements.[15] On culture day 3, the grafts were lifted to the air-liquid interface and the medium was adjusted to increase calcium to 1 mM. On culture day 4, calcium was increased to 1.5 mM, epidermal growth factor was removed, and the grafts were maintained with daily changes of this medium until grafting on day 14 of incubation.

Grafting of Skin Substitutes to Athymic Mice

All animal studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee. CSSs were grafted onto athymic mice (BALB/c, nu,nu, Harlan Sprague Dawley, Inc., Indianapolis, Ind) on CSS culture day 14.[16] Twenty mice per experiment were used in three separate grafting procedures, with four mice per time point. Topical nutrients and antimicrobials were injected into the dressings of each animal on days 1 to 13 after grafting.[16] On day 14 after grafting, the dressings and stenting sutures were removed and the mice were rebandaged from days 14 to 21 with a dressing consisting of N-Terface (Winfield Laboratories, Inc., Richardson, Tex), Xeroform (Sherwood Laboratories, St. Louis, Mo), cotton gauze, and a stretch dressing (Coban; 3M, St Paul, Minn). Grafts were collected on days 1, 3, 7, 14, and 21, and approximately 80% of each graft was flash frozen in liquid nitrogen for quantitative assay, and the remaining fraction was frozen in OCT embedding compound (Miles Laboratories, Elkhart, Ind), and stored at −70°C.

Processing for Enzyme Linked Immunosorbent Assay

All tissue handling was performed at 4°C to keep specimens cold at all times. Enzyme linked immunosorbent assay (ELISA) was performed immediately after processing of each tissue from its frozen state. Individual tissue samples ranging from 0.1 to 1 g dry weight were pulverized in a Bessman Stainless Steel Tissue Pulverizer (Fisher Scientific, Pittsburgh, Pa) chilled previously with liquid nitrogen. Each pulverized sample was resuspended in 4°C phosphate-buffered saline.

For in vitro analysis, human fibroblasts and human keratinocytes from the same strains used to make the CSS were grown to 80% confluency in 100-mm dishes. All medium was removed and stored at 4°C for further analysis. Ice cold phosphate-buffered saline was added to each dish (2 mL/dish) and placed on a rocker at 4°C for 10 minutes. Cells were then harvested using a cell scraper.

All samples (in vitro and in vivo) were homogenized using an Ultra Turrax T25 tissue homogenizer (Janke & Kunkel, IKA-Works, Inc., Cincinnati, Ohio) on ice for 45 seconds and sonicated on ice for 45 seconds with a Fisher Scientific 550 Sonic Dismembranator. Samples were subjected to low-speed (2500 rpm for 20 minutes at 4°C) and high-speed centrifugation (29,000 rpm for 30 minutes at 4°C), stored overnight at 4°C, and analyzed the next morning.

Measurement of Cytokines by ELISA

Samples were kept cold until immediately before the assay. Individual Quantikine kits (R&D Systems, Minneapolis, Minn) for IL-1α, IL-6, and bFGF were used for ELISA, and each sample was run in duplicate. A protein assay using the Bio-Rad Protein Micro Assay procedure[17] was performed concurrently with the ELISA.

Immunohistochemistry

Tissue samples used for immunohistochemical staining were prepared from cryostat sections of unfixed, fresh-frozen excised skin from wound sites at days 1, 3, 7, 14, and 21. The localization of cytokines after grafting was determined using routine procedures for indirect immunofluorescence. Six-μm thick sections were air-dried, fixed in acetone at −20°C for 15 minutes, and air dried again. Sections were rehydrated and incubated with either rabbit anti-human IL-1α polyclonal antibody (1:500; Cistron Biotechnology, Pine Brook, NJ), rabbit anti-human IL-6 polyclonal antibody (1:20; Genzyme, Cambridge, Mass) or rabbit anti-human bFGF (1:100; Sigma Immunochemicals, St. Louis, Mo). The secondary antibody used was goat anti-rabbit IgG phycoerythrin (1:300; Molecular probes, Inc., Eugene, Ore). Sections were examined with a Nikon Microphot-FXA (Nikon Corp., Melville, NY) equipped with an epifluorescence illumination system. Controls were performed by replacing the primary antibody with Tris-buffered saline.

Data Analysis

Three separate grafting procedures were performed. Each data point represents a pooled sampling of one to four mice from two or three different grafting procedures. Each ELISA was performed in duplicate. Mean values are expressed as picograms of cytokine per milligram of final protein yield for each sample, or picograms of cytokine per milliliter in media samples.

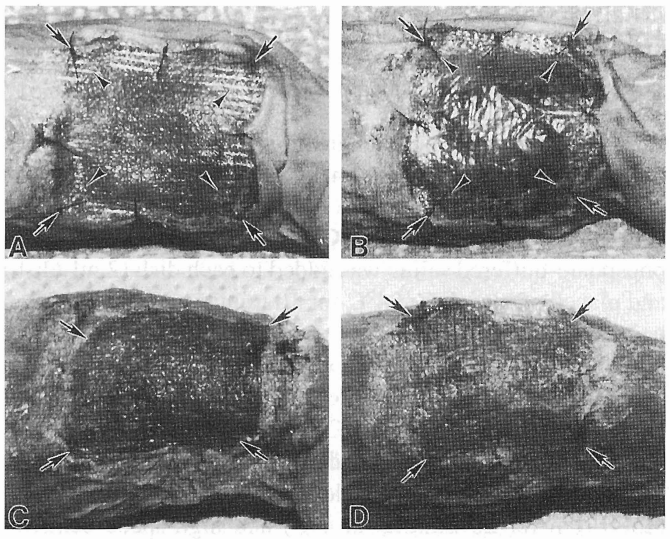

FIG 1. **Healing of CSS after grafting to full-thickness wounds in athymic mice. (*A*) Day 1 after grafting. The epidermis is extremely fragile and weakly adherent to the wound. (*B*) Day 3 after grafting. The epidermis is still fragile with moderate adherence to the wound. (*C*) Day 7 after grafting. The CSS shows signs of vascularization and the epidermis is becoming thicker. (*D*) Day 14 after grafting. The epidermis is thick with a well-formed keratinized layer that readily repels water. Healed skin at day 21 after surgery is similar to day 14 and is not shown. CSS perimeter (*arrows*) and N-Terface dressing (*arrowheads*) are indicated. Magnification, 1.4 ×.**

RESULTS

Animals

Overall survival for the athymic mice was 90%, and mortality that occurred during the first 24 hours after grafting was most probably secondary to anesthetic complications. Overall graft take was greater than 90%. At day 1, the epidermis was extremely fragile and weakly adherent to the wound bed. By day 7, the CSS showed signs of vascularization, and by day 14 the epidermis was thick with a well-formed keratinized layer that readily repelled water (Fig. 1).

In Vitro Expression

The cultured fibroblasts used to make the CSS contained relatively high levels of bFGF (1473 pg/mg) and minimal amounts of either IL-1α (1 pg/mg) or IL-6 (49 pg/mg) (Table 1). Cultured keratinocytes contained 67% less bFGF than was found in the fibroblasts, and high levels of IL-1α (9323 pg/mg). Minimal amounts of IL-6 were produced by the keratinocytes or fibroblasts. No bFGF or IL-1α were found in the medium of the fibroblasts, and only 4 pg/mL was detected for IL-6. The keratinocyte medium contained bFGF (127 pg/mL), but minimal levels of IL-6 (2 pg/mL) were found and no detectable levels for IL-1α.

TABLE 1. **Cytokine levels in vitro**

	bFGF (pg/mg)	IL-1α (pg/mg)	IL-6 (pg/mg)
CSS in vitro	1963	9400	410
Fibroblasts	1473	1	49
Keratinocytes	500	9323	14
	bFGF (pg/mL)	**IL-1α (pg/mL)**	**IL-6 (pg/mL)**
CSS medium	0	7.5	130
Fibroblast medium	0	0	4
Keratinocyte medium	127	0	2

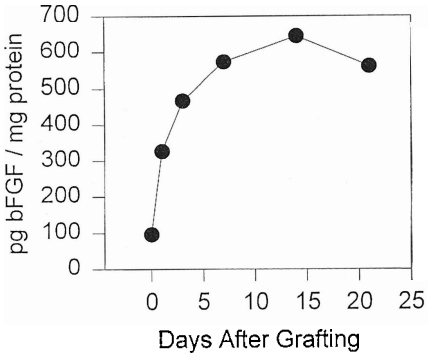

FIG 2. **Plot of the concentration of bFGF versus days after grafting of CSS onto athymic mice from day 0 to day 21. Day 0 is a measure of uninjured mouse skin.**

In vitro, CSS produced both bFGF (1963 pg/mg) and IL-1α (9400 pg/mg) in large quantities. The findings from the individual cultured cell populations suggest that bFGF was derived predominately from the fibroblasts, and IL-1α from the keratinocytes. Although little IL-6 was produced by either fibroblasts or keratinocytes alone, if combined in the CSS, relatively large quantities (410 pg/mg) were produced. These findings suggest that both cell types interact synergistically to produce this cytokine. In addition, IL-6 (130 pg/mL) and IL-1α (7.5 pg/mL), but no bFGF, were detected in the medium of the CSS.

In Vivo Expression

CSSs grafted onto athymic mice were harvested to determine whether cytokine expression was related to results in vitro, and to observe any trends of cytokine expression in wounds over time. bFGF values after grafting were generally lower than in vitro. Levels of bFGF increased 3-fold with the grafting procedure over that of uninjured murine skin. From day 1 through day 21, the range of bFGF was 326 to 644 pg/mg. bFGF increased steadily from day 1 to day 7, and remained at elevated levels until day 21 (Fig. 2). Similarly, bFGF was detected by immunohistochemistry at all of the timepoints, with cytoplasmic staining in nucleated keratinocytes (basal and spinous layers) in the epidermis during the earlier time points (Fig. 3*A*). By day 21, increased staining for bFGF in the basal cell layer of the epidermis was observed. All controls for the immunohistochemistry yielded the expected negative results for each cytokine (Fig. 3*D*).

After grafting, IL-1α increased several fold over uninjured murine skin, but was greatly reduced compared to CSS in vitro. Increased levels were detected at day 3, after which decreased levels were measured at days 7 and 14 (Fig. 4). Elevated levels at day 3 coincided with the clinical observations of revascularization in the grafted CSS, between days 3 to 7. Increased levels of IL-1α were also detected at day 21. From day 1 to day 21 the range of IL-1α was 492 to 1366 pg/mg. Staining for IL-1α by immunohistochemistry also showed greatest signal intensity at days 3 and 21. Positive staining was observed predominately in the nucleated cells of the basal and spinous layers in the epidermis (Fig. 3*B*).

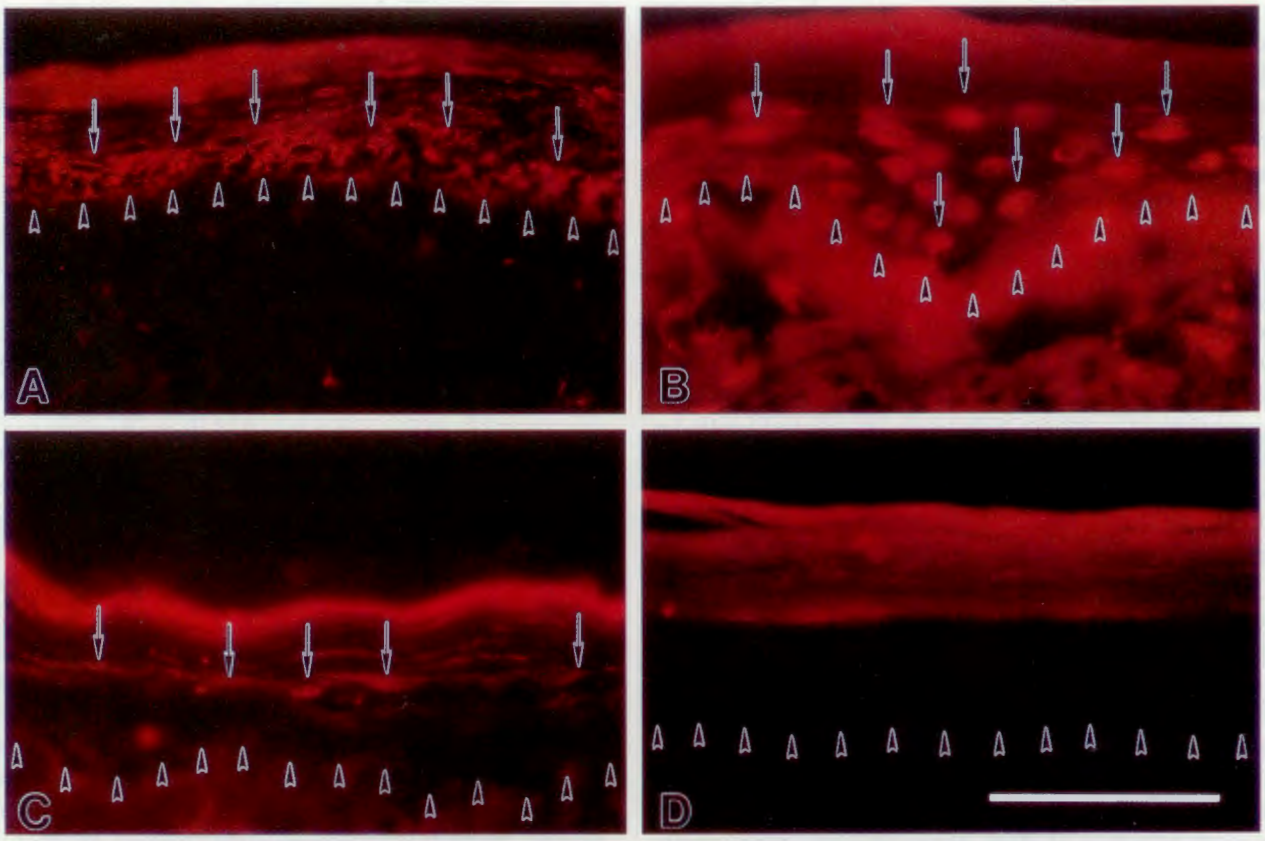

FIG 3. **Immunohistochemical localization of cytokines in healed epidermis after grafting to athymic mice. Dermal-epidermal junction is indicated by *arrowheads* in all micrographs. (*A*) bFGF staining is localized to plasma membranes and cytoplasm of basal keratinocytes (*arrows*). (*B*) IL-1α staining of basal cells and spinous cells in the epidermis is perinuclear (*arrows*). (*C*) IL-6 staining in linear bands extending suprabasal from the spinous layer to the stratum corneum (*arrows*). (*D*) Control section not incubated in primary antibody. Bar, 80 μm.**

Levels of IL-6 were at least 4.5-fold greater in CSS in vitro than values obtained in the grafted CSS. IL-6 also increased steadily after grafting, with greatest concentrations occurring at day 7, followed by a gradual decline to day 21 (Fig. 5). The range of IL-6 from day 1 to day 21 was 27 to 90 pg/mg. Detection of IL-6 by immunohistochemistry showed a linear pattern of staining in the spinous and granular layers of the epidermis (Fig. 3*C*). Similarly to ELISA data, greatest staining for IL-6 occurred in CSS samples at day 7 after grafting.

DISCUSSION

CSSs have been shown to promote wound closure in acute[2,18] and chronic wounds.[19–22] In chronic wounds, stimulation of healing is attributed, in part, to cytokines that are released from keratinocytes that are "activated" in culture.[23–24] Among these cytokines are bFGF, a potent angiogenic factor,[25] and IL-1α and IL-6, which potentiate inflammation.[8,26] Quantitative and qualitative data presented in this report support the hypothesis that keratinocytes directly stimulate wound healing. Systemic release of large amounts of IL-1α after skin injury[27–28] has also been implicated in systemic responses to trauma.[29] Detection of bFGF in healing wounds is consistent with induction of vascularization by skin grafts on excised wounds. In contrast to interleukins, bFGF is not elevated systemically after release in wounds because it is bound with high affinity to sulfated

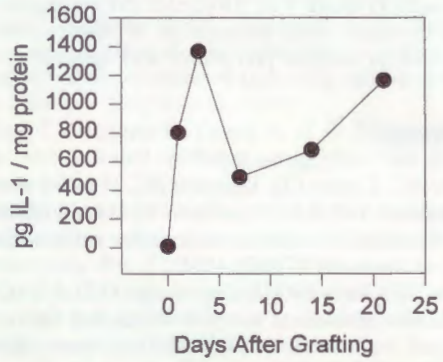

FIG 4. **Plot of the concentration of IL-1α versus days after grafting of CSS onto athymic mice from day 0 to day 21. Day 0 is a measure of uninjured mouse skin.**

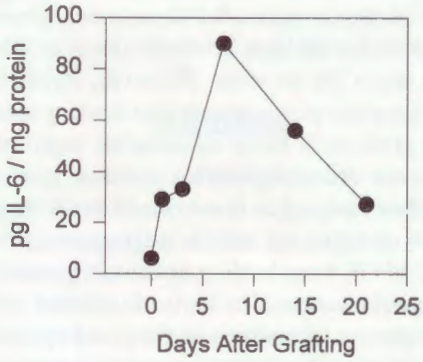

FIG 5. **Plot of the concentration of IL-6 versus days after grafting of CSS onto athymic mice from day 0 to day 21. Day 0 is a measure of uninjured mouse skin.**

glycosaminoglycans of tissue matrix to localize the angiogenic response.[3] The factors studied here are only a small sampling of an extensive network of keratinocyte factors including soluble peptides (growth factors, cytokines, proteases), lipids (arachidonic acid metabolites), and matrix molecules (basement membrane) that contribute directly to all phases of the wound-healing process.[30,31] A similar but distinct network of cytokines has been described for fibroblasts.[32] Both cytokine networks act by autocrine,[33] paracrine,[7,23] and endocrine[27] mechanisms to regulate the process of wound healing.

Quantitative analysis of cytokines after grafting of CSSs demonstrates three basic findings. First, ranges of absolute concentrations of cytokines tested here are greatest for IL-1α (492–1366 pg/mg), followed by bFGF (326–644 pg/mg) and IL-6 (27–90 pg/mg). Because the molecular weights of IL-1α and bFGF are very similar (17 kd), levels detected represent greater molar proportions of IL-1α (8.2×10^{-14} moles/mg) than of bFGF (3.8×10^{-14} moles/mg). With a molecular weight of 20.5 kd, IL-6 has the lowest concentration (4.4×10^{-15} moles/mg) of these three cytokines in this model. Second, maximal levels of each factor occur in a temporal sequence with IL-1α at day 3 after grafting, followed by IL-6 at day 7 and bFGF at day 14. These temporal maxima agree with other studies[27,34,35] that identify relatively rapid increases of circulating IL-1 after burns and bacteremia with subsequent increases of IL-6. bFGF also increases rapidly during the early response to burn injury.[3] These results suggest expression of these cytokines is regulated differentially during wound healing. More rapid increases and decreases of interleukin levels suggest that the half-life of these agents in wounds may be shorter, and that turnover may be more rapid. Third, levels of each of these cytokines in skin substitutes before grafting is substantially greater than after grafting, suggesting that different mechanisms of expression act in vitro. Relatively high levels of these peptides in vitro suggest that either more up-regulation and/or less down-regulation of expression occurs in vitro. Non-native levels of cytokines in vitro would not be surprising considering that many aspects of keratinocyte physiology (e.g., barrier formation, basement membrane, pigmentation) are abnormal in vitro.[13,36,37]

Cytokines evaluated in this study have distinct roles in wound healing and are expressed in epidermis. bFGF is localized to basal keratinocytes by immunofluorescence staining, with only minimal labeling of connective tissue cells. This finding is somewhat unexpected, considering that bFGF is detected at high concentrations in both cultured fibroblasts and CSS in vitro. However, strong staining of epidermal keratinocytes suggests that healing epidermis may establish a gradient of bFGF to stimulate ingrowth of vasculature up to the dermal-epidermal junction. It may be speculated that fibroblasts act as reservoirs of bFGF that is released at the time of injury to initiate angiogenesis,[38] and that a gradient of bFGF from healing epidermis promotes completion of vascularization. IL-1α is distributed in a distinct perinuclear pattern of staining in basal and spinous keratinocytes after grafting. In vitro, nanogram levels are detected in keratinocytes and CSSs. Together, these results suggest that the majority of IL-1α production of parenchymal cells in healing skin is derived from keratinocytes. These findings are consistent with the hypothesis that keratinocytes are central to the initiation of inflammation in wound healing in skin.[8,30] Antibody to IL-6 stains the keratinocytes weakly in the suprabasal strata of epidermis. This may reflect the relatively low molar concentrations of IL-6 detected by ELISA. IL-6 was detected in medium for CSS, suggesting that it is released by cells in the cultured graft. In addition, there was a synergistic increase of IL-6 detection in CSS relative to keratinocytes or fibroblasts separately. This finding suggests action of a paracrine mechanism for expression of IL-6 between keratinocytes and fibroblasts. However, it cannot be determined from this study which of these two cells might be upregulated by the presence of the other, or whether both cell types increase IL-6 synthesis.

This preliminary study was performed to determine whether cytokine peptides could be measured directly in a model of wound healing with cultured skin substitutes. Although that objective was accomplished, levels of bFGF, IL-1α, and IL-6 were not measured in native human or mouse skin as control grafts on athymic mice. Also, experiments reported here were performed only two or three times. Therefore, whether statistical differences exist among experimental conditions could not be determined, and additional experiments are required to strengthen the findings of this study. Finally, qualitative and quantitative analyses of other mediators of wound healing by detection of peptides will depend on the relative abundance and stability of each factor during wound healing. Factors with greater abundance and stability will be more subject to quantitative evaluation.

Determination of cytokine levels in CSS before and after grafting is expected to contribute to regulation of cytokine expression by CSS in vitro, and to understanding of the factors that mediate inflammation after application of CSS to wounds. By optimizing expression of factors that are critical to wound healing, it is expected that engraftment and healing of CSS on wounds will become more rapid, and more complete. Improved healing of acute and chronic wounds with CSSs may reduce mortality and morbidity from traumatic injuries and degenerative skin diseases.

Acknowledgments

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DISCUSSION

Dr. Anthony A. Meyer (Chapel Hill, North Carolina): I'd like to thank the authors for a very early manuscript that allowed me to review it. I'd also like to thank Dr. Goretsky for a very nice presentation.

I think the authors have undertaken to analyze the production of these three cytokines important to wound healing, both in vitro and in vivo, measuring specimens of a CSS. This CSS is a combination of inert C-GAG matrix embedded with

human fibroblasts and covered with human keratinocytes. It's a preparation that's been studied for several years.

The healing of wounds involves a somewhat organized set of events and messages delivered by cytokines that may in fact progress in a sometimes very disorganized manner, and that result in a stimulation of both proliferative and phenotypic expression of certain cell populations that should lead to wound healing.

This study analyzed the appearance of three of these cytokines, IL-1α, IL-6, and bFGF as a means of determining the effects of these cytokines on wound healing. The results suggest that there's a fairly predictable pattern for production of these cytokines at different points in a 21-day healing wound.

I had a few methodologic questions and a few comments. With respect to the system: First, did you determine if there was any cross-reactivity of your anti-human cytokine antibodies with murine cytokines? This is especially important. In experiments in vitro, some of the areas where you saw fluorescence would obviously not have been human cells or tissue at that level.

Additionally, was there any evidence of cross-reactivity with any bovine pituitary extract or anything else in the culture that may have helped light up some of your grafts?

Second, was there consistent time length between the preparation of samples and culture media assays? Given the dramatic difference between the levels of cytokine in both the cells and the culture media, there seems to be a large gradient.

And with respect to interpretation of the results, I have the following questions and comments. Given this dramatic gradient between the amount of the cytokines found in intercellular extracts and culture media, do you feel that this represents an autocrine effect primarily targeted toward the cell of production, or do you feel that there is any limited paracrine effect from freely diffusing cytokines?

In addition to that question, have you considered looking at the RNA message for those cytokines in those individual cell populations to see if this represents increased effectiveness in translation or greater gene expression?

You demonstrated synergistic increase in cytokines when the cell populations were mixed together. Did you consider growing these cell populations in the absence of the inert matrix to see if that participates significantly in this amplification?

And finally, do you feel that the manipulation of the wound-healing environment will yield a superior wound or one that may close faster or have more collagen, but in effect be less practical when it does not receive the continued feedback of the natural environment?

Dr. Jorge L. Rodriguez (Ann Arbor, Michigan): A very nice presentation. I have two questions concerning the in vivo data. Do you have any measurements of systemic cytokines during the time of grafting or in grafting of the cultured epithelral autografts?

And second, we all know the immunohistochemistry localizes the protein, but do you have any in situ or any mRNA data to clearly allow you to conclude that local cytokines or the production are not secondary effects of the systemic circulation?

Dr. Michael Goretsky (closing): I want to thank Dr. Meyer for reviewing our paper, and for both Dr. Meyer's and Dr. Rodriguez's comments.

In response to Dr. Meyer's first question regarding the cross-reactivity of the antibodies, they were human-specific antibodies. For the immunohistochemistry, you can see that the background reactivity of specific antibodies was minimal. We believe that the predominant staining seen in the epidermis and the negligible staining seen in the dermis results from high survival of cultured keratinocytes, and low survival of dermal fibroblasts in the skin substitute after grafting.

Regarding the cross-reactivity in the ELISAs, we did not do any specific test for that. These antibodies are characterized as human specific, although there is sometimes slight cross-reactivity with native murine skin or higher background values than expected. This is why murine skin was included as a control.

Culture media were collected simultaneously with the cell populations each time we performed this experiment. So, time intervals for collection of media and cells were always the same.

With respect to the question about autocrine versus paracrine regulation of these cytokines, we believe it is probably a combination of the two. Which mechanism is more predominant is difficult to interpret from these preliminary studies.

In response to questions from both Dr. Meyer and Dr. Rodriguez regarding expression of RNA message, this is a preliminary report and not unexpectedly, more questions were raised than answered. At present, we are hoping to proceed with RNA analysis and in situ hybridization to address those questions.

Regarding co-culture of keratinocytes and fibroblasts without a matrix, that is a good suggestion and something that we can investigate.

And with regard to testing of systemic cytokines, that was beyond the scope of this preliminary study, but something that we recognized in retrospect and are planning to do in the next series of studies.