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Characterization of Growth Hormone Enhanced Donor Site Healing in Patients with Large Cutaneous Burns

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Background

Human growth hormone is an anabolic agent that attenuates injury-induced catabolism and stimulates protein synthesis. Recombinant human growth hormone (rhGH) administered therapeutically to patients with massive burns has been shown to increase the rate of skin graft donor site healing. It has been postulated that growth hormone affects wound healing and tissue repair by stimulating the production of insulin-like growth factor-1 (IGF-1) by the liver to increase circulating IGF-1 concentrations. The mechanism by which it improves wound healing, however, remains in question. The authors hypothesize that rhGH up-regulates IGF-1 receptors and IGF-1 levels both systemically and locally in the wound site to stimulate cell mitosis and increase synthesis of laminin, collagen types IV and VII, and cytokeratin. This hypothesis was tested in nine patients with burns covering >40% of total body surface area.

Objective

The authors assessed the efficacy of rhGH in promoting several major building materials in the donor site of patients with massive burns.

Methods

Ten massively burned patients with full-thickness burns covering more than 40% of total body surface area were participants in a placebo-controlled prospective study to determine the efficacy of 0.2 mg/kg/day rhGH on donor site wound healing and to identify some of the major components involved in wound healing and its integrity.

Results

Donor sites in burn patients receiving rhGH showed an increased coverage by the basal lamina of 26% for placebo to 68% coverage of the dermal-epidermal junction. Insulin-like growth factor-1 receptors and laminin, types IV and VII collagen, and cytokeratin-14 all increased significantly. Healing times of the donor sites were significantly decreased compared with patients receiving placebo.

Conclusion

Results indicate that growth hormone or its secondary mediators may directly stimulate the cells of the epidermis and dermis during wound healing to produce the structural proteins and other components needed to rebuild the junctional structures.

Human growth hormone has been shown to be an anabolic agent that attenuates injury-induced catabolism, stimulates protein synthesis and immune function, and increases muscle mass.[1-4] Recombinant human growth hormone (rhGH) administered therapeutically to massively burned patients has been shown to increase the rate of skin graft donor site healing by 20% to 30%, resulting in a 25% to 35% reduction in the length of hospital stay.[5,6] It has been postulated that growth hormone affects wound healing and tissue repair by stimulating the production of insulin-like growth factor-1 (IGF-1) by the liver to increase circulating IGF-1 concentrations.[7-11] However, the mechanism by which it improves wound healing remains in question. We hypothesize that rhGH up-regulates IGF-1 receptors and IGF-1 levels both systemically and locally in the wound site to stimulate cell mitosis and increase laminin, collagen types IV and VII, and cytokeratin synthesis. This hypothesis was tested in nine patients with burns covering >40% of total body surface area.

METHODS

The ten patients selected for study ranged from 6 to 30 years of age, with full-thickness burns covering more than 40% of total body surface area. All patients were resuscitated by a standard formula, with intravenous fluids administered to maintain a urinary output of 0.5 to 1.0 mL/kg/hour. Electrolyte supplementation was given to achieve appropriate serum concentrations,[12] and nutritional support was given enterally at 6300 kJ/m^2/body surface area/day plus 6300 kJ/m^2/total body surface area burn/day.[13-15] One of two surgeons excised and grafted the burn wound, excluding the face and perineum, within 48 hours of admission. Donor sites were harvested using a Padgett electric dermatome (Padgetts Instruments, Kansas City, MO) set at 0.010 inches and dressed with Scarlet Red (Sherwood Medical, St. Louis, MO) impregnated fine mesh gauze. From the third postoperative day, the donor site was examined by one of two evaluators blinded to the treatments. Using sterile technique, each corner of the gauze was gently lifted, using forceps to determine the adherence of the dressing to the underlying tissue. Any unattached dressing was trimmed away. This procedure was repeated daily until the gauze no longer adhered to the underlying wound. Healing time was defined as the time, in days, for the first

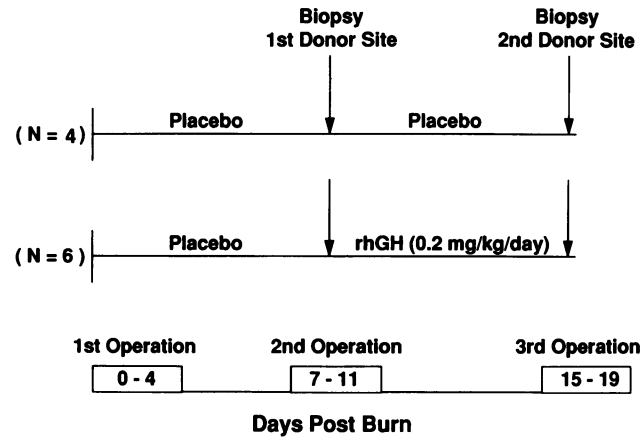

Figure 1. Schematic of the therapy and biopsy protocol.

or second donor site to heal as indicated by atraumatic removal of the Scarlet Red gauze.

All biopsy samples were taken from split-thickness skin graft donor sites on the thigh. Each patient served as his or her own control. Seven days after the first grafting procedure (postburn days 7–11), a re-epithelialized donor site was sampled by infiltrating the region with 1% lidocaine and excising a small strip of skin (10 mm × 2 mm) with sharp dissection. Six patients were started on 0.2 mg/kg/day rhGH by subcutaneous injection at the time of the second split-thickness skin harvest. Seven days later, the second donor site was sampled (postburn days 15–19). Each harvest was taken from normal skin areas on the lateral thigh. Four patients not treated with rhGH also had biopsies taken from their first and second donor sites. Figure 1 depicts the scheduled procedures. Biopsy specimens were divided into three portions: one fixed in 10% phosphate-buffered formalin and embedded in Paraplast (Oxford Labware, St. Louis, MO) for histologic study; one fixed overnight in cold 2% sodium cacodylate-buffered glutaraldehyde, postfixed for one hour in 1% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin for electron microscopic study; and one embedded in O.C.T 4583 compound (Miles Tissue-Tek, Elkhart, IN) and rapidly frozen in melting 2-methylbutane (isopentane) for preparation of cryostat sections.

For electron microscopy studies, 0.5-μm-thick sections were stained with toluidine blue to identify the areas of interest; and ultrathin (70-nm) sections were prepared using a diamond knife and stained with uranium acetate and lead citrate. Electron micrographs were prepared at constant magnification to include all of the dermal-epidermal junction seen using a single specimen grid. On 8 × 10″ prints, the dermal-epidermal junction was marked, and the lamina densa of the basal lamina was traced where visible. Grazing sections of the dermal-

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epidermal junction were not included in the measurement. The lines were traced into computer memory using a Summagraphics tablet with appropriate calibration, and their length measured using Sigma Scan software (Jandel Scientific, Corte Madera, CA). Results were expressed as the percent of the total linear measure of the dermal-epidermal junction where lamina densa was visible.

Light microscopic immunoperoxidase was used for IGF-1 receptor, cytokeratin-14, laminin, and type IV collagen using 6-μm paraffin sections, and for type VII collagen using 6-μm frozen sections. Paraffin sections for study of IGF-1 receptor or cytokeratin-14 (CK14) were treated with 0.3% hydrogen peroxide in methanol for 20 minutes to inactivate endogenous peroxidase, treated with 5% dry milk for 2 hours and with blocking serum for 20 minutes, and then incubated overnight at room temperature with an appropriate dilution of biotinylated primary antibody. For localization of laminin and type IV collagen, the procedure was modified to include predigestion of the sections with pepsin, (0.1% in 0.5 mol/L acetic acid for 2 hours) and incubation in a solution of polyoxyethylenesorbitan (Tween 20, Sigma Chemical Co., St. Louis, MO) and 0.1% bovine serum albumin in tris-buffered saline for 20 minutes. For labeling of type VII collagen, cryostat sections were incubated in 1% sodium periodate for 20 minutes and 0.1 mol/L ammonium chloride for 20 minutes before incubation in primary antibody. Immunolabeling was detected by using commercial reagents for avidin-biotin-peroxidase complex staining (Vector Laboratories, Burlingame, CA) with diaminobenzidine as substrate, by intensifying the reaction product with brief exposure to 1% osmium tetroxide, and then by lightly counterstaining with hematoxylin and eosin. Controls included omission of primary antibody and substitution of nonimmune immunoglobulin G. The antibodies used were rabbit antihuman IGF 1-receptor, α subunit (Upstate Biotechnology, Lake Placid, NY), at 5 mg/mL, polyclonal antibody to CK14 (Biogenex, San Ramon, CA, prediluted), affinity purified rabbit antilaminin (E-Y Laboratories, San Mateo, CA) at 10 mg/mL, rabbit antibody to type IV collagen (Biogenex, San Ramon, CA) at 6 mg/mL, and murine monoclonal antibody to type VII collagen, clone LH7.2 (Serotec, Harlan Bioproducts, Indianapolis, IN) at 1:100. The intensity of immunostaining was given a score from 0 to 4. Staining scores from three observers, blinded to each other and to the therapy, were averaged for IGF-1 receptors, laminin, cytokeratin-14, and types IV and VII collagen.

Data presented in tables and text are means ± SD. Period I values were compared with period II values using the unpaired t test, $p < 0.05$. Informed consent for the study was obtained, and the procedures followed were approved in accord with the ethical standards of the Committee on Human Experimentation of the University of Texas Medical Branch.

RESULTS

Patient characteristics are depicted in Table 1. Light microscopic study of the biopsies revealed that by 7 days after harvesting each split-thickness skin graft, the epidermis had regenerated to at least normal thickness, and mitoses were rarely observed. By this time, the proliferative phase of re-epithelialization appears to be complete, and differentiation is occurring to form a new junctional apparatus at the dermal-epidermal basal lamina zone and to re-establish keratinization. No substantial differences were seen in cell size or number or the local inflammatory response between the biopsies taken after treatment with placebo or rhGH.

During administration of 0.2 mg/kg/day rhGH, IGF-1 serum concentrations increased threefold when compared with placebo periods and are similar to the IGF-1 levels measured in a previous study.[1] A significant decrease of more than 2 days in donor site healing time was shown for those receiving rhGH compared with their placebo times (Table 2). In four burn patients receiving placebo during both their first and second donor site healing periods, no significant differences could be shown between the first and the second healing times (Table 3).

Electron Microscopy

No differences in keratinocyte size or differentiation were seen among treatment groups. Although the epider-

Table 1. PATIENT CHARACTERISTICS

Period I	Period II	n	Age (yrs)	Sex (F/M)	Wgt (kg)	TBSA Burn (%)	3rd Degree Burn (%)
Placebo	rhGH	6	16 ± 8	2/4	45 ± 17	69 ± 13	55 ± 25
Placebo	Placebo	4	7 ± 3	0/4	30 ± 10	47 ± 9	44 ± 9

TBSA = Total body surface area.
Values are means ± SD.

Table 2. MEAN VALUES FOR HEALING TIMES AND EXTENT OF BASAL LAMINA FORMATION

	Healing Time (Days)	Basal Lamina % Coverage
Period I		
Placebo	7.7 ± 1.2	26 ± 11
Period II		
rhGH	5.6 ± 0.7*	68 ± 16*

* $p < 0.05$ compared with paired value in the absence of rhGH.
Data represent healing times and extent of basal lamina formation as a percent of the total dermal-epidermal junction examined in biopsies from split-thickness skin grafts taken 7 days after harvesting. Normal skin value for basal lamina coverage is 93 ± 7%.
Values are means ± SD; n = 6.

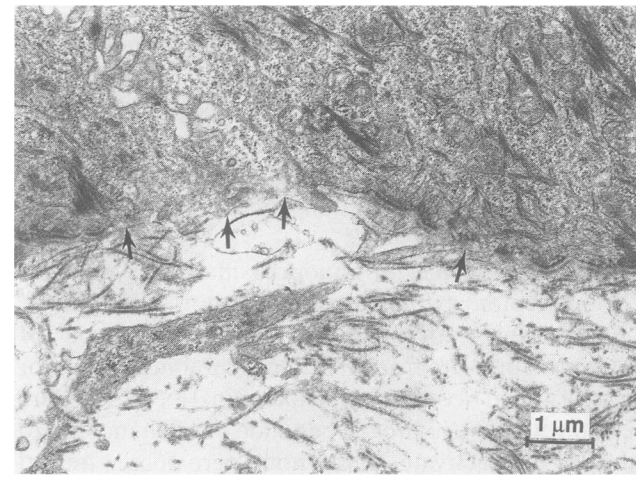

Figure 2. Representative electron micrograph of a placebo-treated donor site biopsy 7 days after harvest. Arrows show gaps in the basal lamina underlying well-differentiated stratified squamous epithelium. Magnification × 17,300.

mis appeared well formed, the lamina densa of the basal lamina was indistinct and discontinuous. Hemidesmosomes were seen only in basal cells already lined by basal lamina. Measurement of the extent of basal lamina production as a percent of the total length of dermal-epidermal junction examined revealed much more extensive formation of new basal lamina during the treatment with growth hormone, averaging 68% ± 16% after the treatment period *versus* 26% ± 11% for the placebo period preceding rhGH therapy (Table 2). No significant difference in the extent of basal lamina formation could be shown between the four burn patients who received only placebo for both treatment periods. Gaps in the basal lamina are shown in Figure 2.

Immunohistochemical Staining

Immunostaining for IGF-1 receptors showed cytoplasmic staining of keratinocytes. An increase in staining intensity, located in the mid epidermis of skin graft donor site biopsy, was observed during rhGH therapy (Fig. 3A).

Immunostaining for laminin revealed relatively faint and discontinuous staining of the dermal-epidermal junction in biopsies of donor sites taken 7 days after grafting without growth factor treatment. There was a significant increase in laminin staining during rhGH treatment (Fig. 3B). Staining for type IV collagen showed an increase in staining of the dermal-epidermal junction during rhGH treatment compared with placebo (Fig. 3C).

Immunostaining for type VII collagen, a major component of the anchoring fibrils associated with the hemidesmosomes, which anchor the epidermis to the dermis, showed a distinct localization in the dermal-epidermal junction in uninjured skin. Faint and variable staining was seen in the biopsies without treatment taken 7 days after harvesting. Staining for type VII collagen increased in intensity after growth hormone treatment, although it still showed prominent variation in staining intensity along the junction, unlike the pattern seen in uninjured skin (Fig. 3D).

Cytokeratin-14 is a relatively low molecular weight keratin species which, in the normal mature epidermis, is restricted in its expression to the basal cells. In all the biopsies examined, CK14 was expressed in the keratinocytes of the middle layer of the epidermis with an increase in staining intensity observed during rhGH treatment (Fig. 3E). In some cases, it was present in all the layers of the epidermis. This change may represent an alteration in the keratinocyte phenotype, with lack of differentiation, or specific differentiation toward the nor-

Table 3. HEALING TIMES AND EXTENT OF BASAL LAMINA FORMATION FOR PLACEBO CONTROLS

	Healing Time (Days)	Basal Lamina % Coverage
Period I		
Placebo	7.3 ± 0.4	44 ± 12
Period II		
Placebo	7.5 ± 0.5	48 ± 17

No significant difference could be shown between treatment period I and II.
Data represent healing times and extent of basal lamina formation as a percent of the total dermal-epidermal junction in biopsies from split-thickness skin grafts taken 7 days after harvesting.
Values are means ± SD (n = 4).

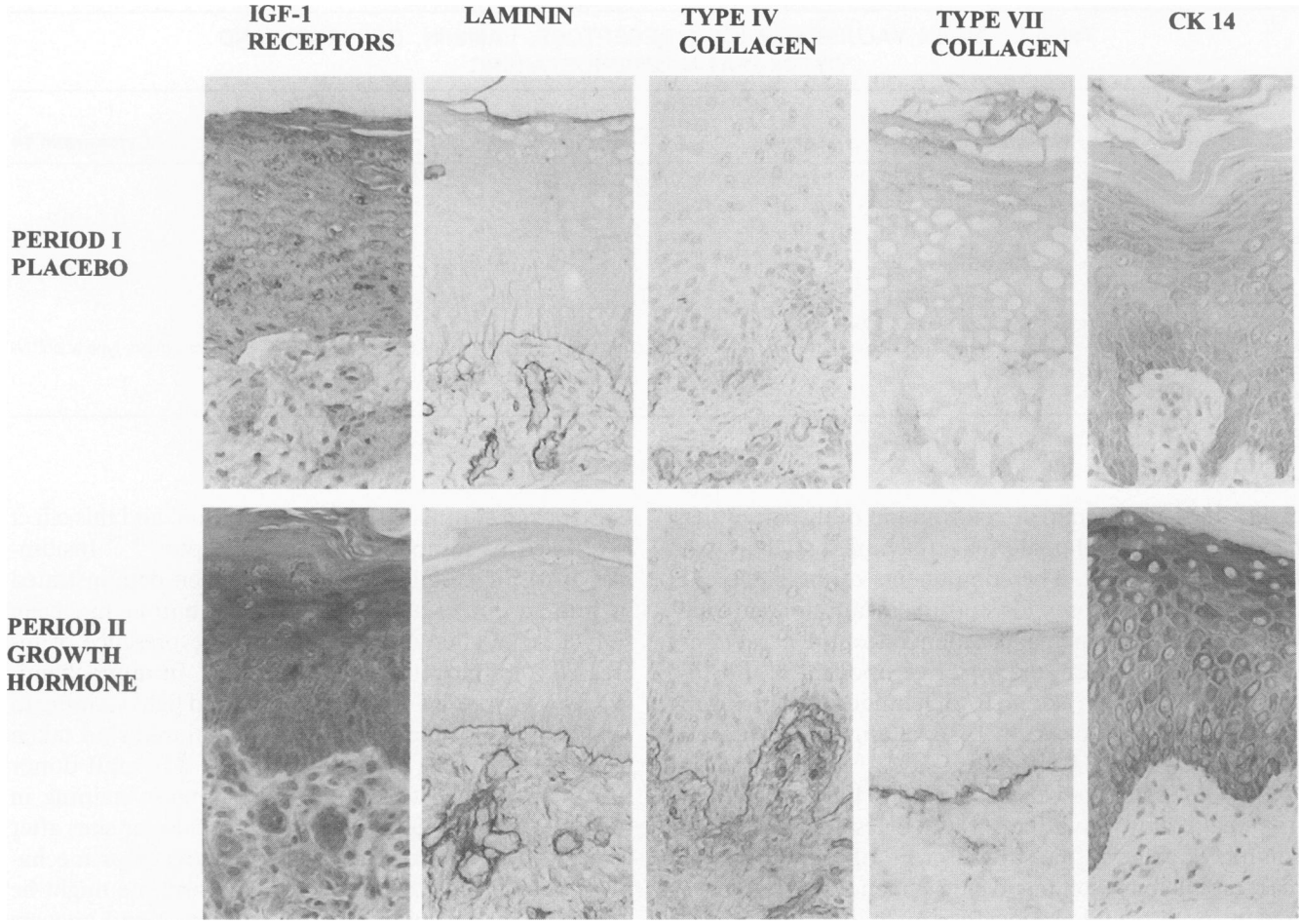

Figure 3. Micrographs for placebo treated (upper) *vs.* rhGH treated (lower) skin graft donor sites showing immunostaining for (A) IGF-1 receptors, (B) laminin, (C) type IV Collagen, (D) type VII collagen, and (E) cytokeratin 14. Magnification × 250.

mal basal cell phenotype. Table 4 summarizes the immunostaining values.

DISCUSSION

We and others have demonstrated that daily administration of rhGH in severely burned patients results in more rapid healing of split-thickness skin graft donor sites and consequently reduces the average length of hospitalization.[2,5] Recombinant human growth hormone also has been shown to enhance wound collagen content and tensile strength in animal experiments, even with a lack of adequate nutritional support.[16,17] This study represents the first effort to examine quantitatively the effects of growth hormone on wound healing in human patients. The study was designed so that each patient served as his own control. Uninjured skin from the thigh was used to prepare a split-thickness skin graft, and a biopsy of the donor site was taken after 7 days. Uninjured skin from another nearby region of the thigh was used to prepare the second split-thickness skin graft, growth hormone was administered daily for 7 days, and another skin biopsy was taken. The control phase preceded the phase of growth hormone treatment, rather than using a crossover protocol, because of concern that residual secondary or tertiary effects of growth hormone administration might persist long after administration of growth hormone was stopped. We chose to examine the healing skin graft donor site 7 days after harvesting, a time when untreated patients would have completed re-epithelialization, less variability in sampling would be anticipated, and the biopsy would only minimally interfere with wound healing. On the basis of our previous work on growth factor effects on regeneration of the tracheal epithelium[18,19] and the known effects of growth hormone,[20] we hypothesized that treatment with growth hormone would be associated with stimulation of mitosis and proliferation of epidermal cells, and that there would be

Table 4. MEAN VALUES FOR IGF-1 RECEPTORS, LAMININ, COLLAGEN AND CYTOKERATIN IMMUNOSTAINING

	IGF-1 Receptors	Laminin	Collagen Type IV	Collagen Type VII	Cytokeratin 14
Period I					
Placebo	0.7 ± 0.4	0.7± 1.1	0.9 ± 1.0	0.4 ± 0.3	0.7 ± 0.2
Period II					
rhGH	2.3 ± 0.4*	2.5 ± 1.5*	2.4 ± 1.3*	2.1 ± 1.5*	2.3 ± 0.5*

* $p < 0.05$ compared with paired value in the absence of rhGH.
Staining values range from 0–4, with 4 being the most intense. Normal skin values of burn patients were 1.0 for IGF-1 receptor staining and 4.0 for laminin, type IV and VII collagen, and cytokeratin-14 staining.
Values are means ± SD; n = 6.

earlier and more complete reformation of the structures necessary for dermal-epidermal attachment after growth hormone treatment. The components of the basement membrane zone chosen for study were laminin and type IV collagen, the principal components of the basal lamina; type VII collagen, the major component of the anchoring fibrils which extend from hemidesmosomes into the dermis; and cytokeratin 14, a keratin species associated with epidermal basal cells.

Previous studies of wound healing in humans and animals have shown that the keratinocytes adjacent to the wound, whether on the surface or in follicles or glands, react rapidly by switching to a new phenotype characterized by motility and phagocytosis, with increased cell division occurring at some distance from the advancing front.[21–25] The specialized attachment structures at the dermal-epidermal junction require several additional days to form, with expression of bullous pemphigoid antigen followed by type IV collagen and laminin, with formation of a basal lamina and hemidesmosomes.[26,27] All the components of the basal lamina can be synthesized by wound keratinocytes, although the connective tissue cells of the matrix also may contribute.[28]

Growth hormone may act directly through its own specific cell surface receptor to stimulate cell division or protein synthesis, may act by stimulating IGF-1 release from the liver into the circulation, or via paracrine or autocrine mechanisms at the injured site.[20,29,30] Local synthesis of several growth-stimulating substances might be enhanced by growth hormone.[31–34] Growth hormone is known to stimulate synthesis of insulin-like growth factor 1 (IGF-1, somatomedin C) in liver and other tissues.[20,35] Growth hormone receptors are present in human skin but are more plentiful in dermal fibroblasts than in keratinocytes.[36,37] The dose of growth hormone given in this study has been shown to be sufficient to produce elevation in serum IGF-1 levels.[5] Insulin-like growth factor-1 is a mitogen for cultured human keratinocytes and stimulates their proliferation, and this effect is blocked by antibody to IGF-1 receptor.[38,39] Insulin-like growth factor-1 receptors have been demonstrated in human epidermis, and their distribution has been shown to be altered in psoriasis in the presence of increased keratinocyte proliferation.[36,40] Immunochemical staining for IGF-1 receptors revealed light staining in the mid zone of the epidermis in uninjured skin taken from burn patients and in biopsies of skin graft donor sites with placebo treatment. More intense staining in the same region was seen in biopsies of donor sites after growth hormone treatment. Thus, the receptor mechanism by which the effects of growth hormone might be expressed in the epidermis remains intact and appears enhanced in burn patients receiving growth hormone therapy.

The junctional adhesion mechanism at the dermal-epidermal junction includes hemidesmosomes, a basement membrane or basal lamina including a lamina lucida and a lamina densa, anchoring fibrils, and tonofilament bundles in epidermal basal cells. In this study, we measured the completeness of the newly formed basal lamina lining the basal cells of the epidermis and found it to be significantly more complete in the biopsies taken after growth hormone treatment. Laminin and type IV collagen are the major protein components of the lamina densa of the basal lamina,[26] and both showed more intense staining after growth hormone treatment. Type VII collagen is the major component of the anchoring fibrils, which extend from the basal lamina at each hemidesmosome around the adjacent fibers of dermal (type I) collagen.[41,42] This component of the junctional adhesion mechanism also showed more intense immunocytochemical staining after growth hormone treatment. The specimens from patients with the greatest improvement in wound healing time after growth hormone treatment also showed the greatest enhancement of immunostaining for laminin and for collagen types IV and VII. Taken

together, these results indicate that growth hormone treatment accelerates the differentiation of the junctional mechanisms necessary for firm dermal-epidermal adhesion. Multiple cytokeratin types are synthesized by epidermal keratinocytes, and the pattern of expression changes as keratinocytes mature. Cytokeratin-14 is expressed exclusively by basal cells in the normal epidermis and constitutes a major protein component of the tonofilament bundles that attach to cell junctions and are thought to play a role in epithelial attachment.[43,19] In all biopsies taken 7 days after harvesting a split-thickness skin graft, CK14 expression was seen most prominently in the mid epidermis, the zone that is thought to represent the proliferative compartment from which newly formed basal cells arise during wound healing.[25] More CK14 expression was seen in biopsies taken after growth hormone treatment. This represents enhanced production of a protein that may have an important role in basal cell attachment.

In this study, biopsies taken from a second skin graft donor site, after treatment with growth hormone, consistently showed more complete differentiation of the dermal-epidermal junctional apparatus. We considered the possibility that improved healing of the second donor site might represent a nonspecific effect because of the patient's systemic responses to the burn injury, or to some aspect of the intensive care provided in the hospital, which may vary with time postburn. To examine this possibility, we studied biopsies taken according to the same protocol in four patients who did not receive growth hormone treatment during the healing phase of either the first or the second skin graft donor site. No differences were observed in the completeness of the newly formed basal lamina. Thus, the changes described here are thought to be effects of growth hormone treatment. It is possible that the changes observed may be secondary to stimulation of keratinocyte proliferation and more rapid re-epithelialization of the wound by growth hormone, allowing the processes of differentiation to begin earlier. Growth hormone or its secondary mediators also may have directly stimulated the cells of the epidermis and dermis to produce the structural proteins and other components needed to rebuild the junctional apparatus. The effects of growth hormone treatment on re-epithelialization may be mediated directly or via local or systemic production of other factors that directly affect epidermal differentiation.

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Discussion

DR. LORING W. RUE III (Birmingham, Alabama): Thank you, Dr. Jurkiewicz and Dr. Copeland, Members and Guests. It is a true pleasure to be asked to discuss this paper, because it represents another in a series of contributions which have enhanced our understanding of the optimization of donor site healing through the use of recombinant human growth hormone.

In previous work by Dr. Herndon's group, growth hormone has significantly enhanced donor site healing time, thereby decreasing the time to definitive wound closure, more quickly reversing the hypermetabolic state and, in more practical terms, shortening the length of hospital stay by as much as 25%.

Cutaneous wound healing requires reconstitution of the dermal and the epidermal junction, and particularly the basement membrane for optimal skin strength and durability. Fibronectin, laminin and collagen Types I, IV and VII all play a role in achieving this wound healing. Dr. Herndon and his colleagues have elegantly documented these macromolecular elements and their enhancement when treated with growth hormone. Particularly impressive are the photomicrographs and immunostaining micrographs of donor site healing with and without growth hormone. The authors have postulated that the wound healing effects, like many of the anabolic effects of growth hormone, are mediated by somatemedins, particularly IGF-1. One of the down sides to the use of this therapy is the potential for a hyperglycemic response which may limit its usefulness.

With those points in mind, I would like to ask Dr. Herndon the following questions:

Would you comment on the number of patients in this study who required exogenous insulin therapy? Since insulin and IGF-1 exert their anabolic effects through similar membrane receptors, do you believe that insulin could be a contributing factor to the results in this study? Do you believe that the same effects might be observed through the use of insulin alone, which is obviously much less costly?

Could you speculate on the apparent complete absence of improved donor site healing time among the three placebo-placebo patients? Though the growth hormone data is impressive, I am surprised that no improvement in healing time was observed for these patients in period 2, a time following definitive closure of much of the burn wound, when I would expect the nutritional balance to be much better.

And, finally, do you believe the introduction of growth factors in a topical fashion may exert a similar effect as that seen with the systemic effects of growth hormone? I raise this point because of the experience with cultured epithelial autografts in which these hormones are present in the nutrient media. Because of the inconsistent results with this therapy, one might postulate that the actual beneficial effects are from topical enhancement of wound healing through the growth factors in the media, rather than the cells themselves.