Mechanical unloading impairs keratinocyte migration and angiogenesis during cutaneous wound healing

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Radek KA, Baer LA, Eckhardt J, DiPietro LA, Wade CE. Mechanical unloading impairs keratinocyte migration and angiogenesis during cutaneous wound healing. J Appl Physiol 104: 1295–1303, 2008. Published February 21, 2008; doi:10.1152/japplphysiol.00977.2007.—Although initially thought to improve an individual’s ability to heal, mechanical unloading promoted by extended periods of bed rest has emerged as a contributing factor to delayed or aberrant tissue repair. Using a rat hindlimb unloading (HLU) model of hypogravity, we mimicked some aspects of physical inactivity by removing weight-bearing loads from the hindlimbs and producing a systemic cephalic fluid shift. This model simulates bed rest in that the animal undergoes physiological adaptations, resulting in a reduction in exercise capability, increased frequency of orthostatic intolerance, and a reduction in plasma volume. To investigate whether changes associated with prior prolonged bed rest correlate with impaired cutaneous wound healing, we examined wound closure, angiogenesis, and collagen content in day 2 to day 21 wounds from rats exposed to HLU 2 wk before excisional wounding. Wound closure was delayed in day 2 wounds from HLU rats compared with ambulatory controls. Although the levels of proangiogenic growth factors, fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF) were similar between the two groups, wound vascularity was significantly reduced in day 7 wounds from HLU animals. To further examine this disparity, total collagen content was assessed but found to be similar between the two groups. Taken together, these results suggest that keratinocyte and endothelial cell function may be impaired during the wound healing process under periods of prolonged inactivity or bed rest.

Chronic or nonhealing wounds have become a significant financial burden on healthcare resources and represent an increasing problem among individuals subjected to extended periods of bed rest. Both Earth-based mechanical unloading and spaceflight studies have uncovered dramatic physiological changes that occur when subjects are exposed to prolonged bed rest or a microgravity environment. Individuals exposed to mechanical unloading, through periods of hypogravity during spaceflight or prolonged bed rest, acclimate to their new physiological state by altering cardiovascular and metabolic systems. Mechanical unloading is associated with reduced plasma volume, cardiac and vascular adaptations, a reduction in exercise capacity, and increased frequency of orthostatic intolerance (25, 41). Various aspects of overall tissue repair have also been assessed following exposure to absolute mechanical unloading conditions during spaceflight and have revealed significant attenuation in the healing of soft tissue, muscle, and bone (8, 19, 30, 49).

Hindlimb unloading (HLU) represents an established animal model for tissue disuse as a means to simulate bed rest through mechanical unloading (39). Mechanical unloading contributes to substantial alterations in the musculoskeletal system, including muscle atrophy, reduced muscle size and strength, and disuse osteoporosis (44, 56). Ligament healing is significantly impaired when the mechanical load is removed following injury, as rats subjected to 7 wk of HLU revealed an impairment of temporal collagen gene expression ligament remodeling compared with ambulatory controls (36), ultimately leading to abnormal mechanical properties and connective tissue morphology. Immunological studies have identified impaired lymphocyte responsiveness and phagocytic capability of neutrophils when normal individuals were subjected to prolonged bed rest (26, 41, 51). It has also been reported that lymphatic function is impaired in rats subjected to mechanical unloading (28). Incidentally, human cutaneous wound healing abnormalities are often observed in diabetic, obese, and aged individuals who experience restricted physical activity (13).

Wound repair involves a complex interaction between cytokines, growth factors, cells, and the extracellular matrix (ECM) throughout three overlapping phases (13, 60). The inflammatory phase represents the initial phase and is distinguished by the deposition of the fibrin clot, platelet degranulation, the infiltration of immune cells, and the robust release of cytokines and growth factors. The subsequent phase, the proliferative phase, entails wound closure, angiogenesis, and matrix deposition, initiated in response to stimulatory factors produced during the inflammatory phase. The remodeling phase is the final phase of wound healing and includes the generation of degradative enzymes, additional matrix proteins, and cross-linking of collagen, processes which can continue for years to months following the initial injury and result in scar formation (5, 13, 52, 60). Ultimately, ~80% of the original integrity of the skin is regenerated on completion of tissue repair during normal physiological wound healing (5). In an uncompromised individual, the wound healing process generally proceeds without complications. However, numerous factors such as age, nutritional status, physical activity, and systemic disease have been associated with abnormal tissue repair (3, 4, 18, 27, 40, 43, 47, 55, 57).

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Recent evidence suggests that weight-bearing physical activity improves the body’s ability to utilize nutrients, improves circulation, and promotes the healing of injured tissue (11, 24, 34). Despite this evidence, there has yet to be a study conducted in which the subject is exposed to mechanical unloading before cutaneous wounding to determine the specific effects of mechanical unloading on the wound healing process. Observations from prior studies suggest alterations in healing with concurrent tissue injury and mechanical unloading. However, the increasing frequency of wound incidence in geriatric individuals and/or patients subjected to previous mechanical unloading emphasizes the necessity for a direct investigation into the effects of prior mechanical unloading on cutaneous wound healing in vivo.

The purpose of this study was to investigate the global alterations in cutaneous wound healing induced following previous mechanical unloading. We hypothesized that animals subjected to mechanical unloading before cutaneous wounding would exhibit an overall delay in wound repair. For these studies, we subjected rats to the HLU model 14 days before excisional wounding. Wounds were then analyzed for wound closure, angiogenesis, and collagen content from both hind limb-suspended and ambulatory control animals. Using this model, we were able to detect significant delays in wound reepithelialization and revascularization, despite normal levels of proangiogenic cytokines and collagen matrix. The data acquired from these studies emphasize the need for novel treatment strategies to optimize wound healing in individuals subjected to prolonged bed rest before cutaneous injury.

MATERIALS AND METHODS

Animal subjects. Animal experimentation was conducted in accordance with the guidelines of the National Aeronautics and Space Administration (NASA) Institutional Animal Care and Use Committee. All experiments were reviewed and approved by the NASA/Ames Research Center Institutional Animal Care and Use Committee and conducted in compliance with the National Research Council’s Guidelines for the Care and Use of Laboratory Animals.

Hindlimb unloading. Mature male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 350 and 375 g were transported to Ames Research Center. On arrival, rats were housed in standard vivarium cages (47 × 26 × 21 cm) lined with corncob bedding and maintained under the following conditions: light cycle, 12 h, light beginning at 0600, and 12 h dark, 23 ± 1°C, and 30–50% humidity. Food and water was available ad libitum. Animals were weighed daily after arrival to monitor body weight for group assignment. The rats were randomly assigned to a single nonselected group (unmanipulated), or either HLU or control ambulatory groups at one of six time points: baseline and 3, 7, 10, 14, and 21 days postwounding. All groups had six rats within each treatment group. HLU animals and their controls were transferred to specialized suspension cages for unloading 3 days before HLU to acclimate to the suspension cages. Rats in the HLU group were then subjected to HLU for 14 days before wounding to allow for physiological acclimation to mechanical unloading. For the HLU model, animals had a castlike apparatus applied to their tail. To allow for free movement about the cage, the cast was attached to a swivel anchored to the top of the cage, permitting 360° range of motion. Animals were suspended in a 30° head down-tilt position (39). Animals were weighed daily to monitor changes in body mass for the duration of the study. For the wounding procedure, all animals used in the study were anesthetized with isoflurane (2.5% in O2). When completely anesthetized, each animal had its dorsum shaved and six full-thickness excisional punch wounds were placed using a 3-mm biopsy punch (Acu Punch, Acuderm, Lauderdale, FL). Animals were returned to the HLU model immediately after recovery from the wounding procedure and remained unloaded until they were killed. At specific time points after injury, animals were killed, and individual wounds were harvested from the pelt using a 5-mm punch biopsy instrument and snap-frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetechical, Tokyo, Japan). For the hydroxyproline, FGF-2, and VEGF analysis, the level was normalized to wet weight by dividing by the average wet weight (mg) as determined for each time point. For each of the different excisional wound analyses, one of the six wounds was randomly selected from each animal, and this single wound was utilized for that unique analysis. Unwounded skin from the nonselected group was also included for baseline levels. Analgesics (buprenorphine, 0.03 ml) were administered subcutaneously immediately following wounding. Antibiotics were not required, as postoperative infection is exceptionally rare in this model system. All animals were killed by decapitation while under anesthesia (isoflurane, 2.5% in O2). Skin samples were immediately excised and frozen in OCT medium or snap-frozen in liquid nitrogen. Whole blood was collected via heart puncture at the time of dissection while rats were under anesthesia to determine any changes in blood volume. The right and left s oleus, gastrocnemius, and tibialis anterior muscle groups were also harvested to confirm the efficacy of the unloading model and identify muscular atrophy as an indication of mechanical unloading.

Analysis of wound reepithelialization. Wound reepithelialization of excisional wounds (n = 6 per group, 1 wound per rat) was measured using histomorphometric analysis of 10-μm tissue sections from the middle of the wound as described previously (48). Briefly, sections were stained with hematoxylin and eosin. The percentage of reepithelialization [(distance covered by epithelium/distance between wound edges) × 100] was calculated for each section and performed blinded. The average reepithelialization between two wound sections for each animal was used as a unique value. Instead of using a separate group, we generated day 2 wounds by administering two of six wounds 1 day after the initial four wounds were made on rats assigned to the day 3 wound group, since reepithelialization analysis was the only analysis that required a day 2 time point.

Analysis of wound collagen content by hydroxyproline quantification. Hydroxyproline is a major component of the protein collagen and, thus, is used as an indicator to determine collagen levels in tissue. The hydroxyproline content of single individual wounds (n = 6 per group, 1 wound per animal) was determined according to a standard protocol (59). Each wound was harvested from the pelt using a uniform 5-mm biopsy punch. Because the specimens do contain some adjacent normal skin, this analysis compares relative differences between treatment groups rather than absolute values. Since the amount of normal skin is nearly identical in all samples, any observed differences were expected to reflect that of the wound and not normal skin. All reagents for this assay were purchased from Sigma Chemical (St. Louis, MO). Briefly, frozen tissue was hydrolyzed in 2 ml of 6 N HCl overnight at 110°C. The reaction was neutralized with 2.5 N NaOH and diluted 40-fold with MilliQ water. One milliliter of a 0.05 M chloramine T solution was added to 2 ml of the neutralized/diluted solution and incubated for 20 min at room temperature. One milliliter of 3.15 M perchloric acid was added, and the solution was incubated for 5 min at room temperature. One milliliter of 20% p-dimethylamino benzaldehyde was subsequently added, and the mixture was incubated for 20 min at room temperature. The samples were then cooled with cold tap water. The amount of hydroxyproline was determined by comparison to a standard curve measured spectrophotometrically at an absorbance of 557 nm. Samples were analyzed in duplicate.

Analysis of wound angiogenesis. Wound vessel density (n = 6 per group, 1 wound per animal) was determined following immunohistochemical staining with an antibody specific to platelet-derived endothelial cell adhesion molecule-1 (PECAM-1 or CD-31) (PharMingen, San Diego, CA), a marker for vascular endothelial cells, as previously.
formalin solution for 48 h. Formalin-fixed tissues were processed
routine, embedded in paraffin, sectioned at ~5 μm, stained with
hydrogen peroxide in methanol to block endogenous peroxidase activity, and then blocked
specifying binding with 50 μl normal mouse serum (1:100; Sigma). Secondary antibody (biotinylated mouse-α-rat IgG, Jackson, West Grove, PA) incubations were performed for 30 min each.
Sections were subsequently incubated for 30 min with avidin-biotin
nonspecific binding of 3% hydrogen peroxide to block endogenous peroxidase activity, and then blocked
sections were counterstained with Harris hematoxylin. Image Analy-
sections were fixed in 10% neutral buffered formalin for 6 h. Following the

Dermal FGF-2 and VEGF levels were determined using a human FGF-2 ELISA kit or murine VEGF164 (mVEGF164) ELISA kit (Quantikine R&D Systems, Minneapolis, MN) as previously described (53). The amount of murine VEGF164 (mVEGF164) ELISA kit (Quantikine R&D Systems, Minneapolis, MN) as previously described (53). The amount of

Histopathological analyses. Wounds from control or HLU rats

Male rats initially weighing between 350 and 375 g were randomly assigned to

Statistical analysis. Data were analyzed using GraphPad Prism, version 2.1 (GraphPad Software, San Diego, CA). The means and SE

RESULTS

Body mass during experimental period. Male rats initially

Male rats initially weighing between 350 and 375 g were randomly assigned to
either control or HLU groups (30 rats per group) and then further subdivided into five wounding groups (6 rats per group) as described. A timeline of unloading events and tissue collec-
tion is depicted in Fig. 1. Initial body mass of the rats (baseline) did not differ between assigned groups in standard vivarium cages (Fig. 2). Rats were then placed in unloading cages 3 days before unloading or control conditions to acclimate to the specialized cage, during which body mass gain was similar between groups. After 3 days in unloading cages, rats were subjected to HLU or remained ambulatory. During the 14-day unloading and up to 3 days postwounding, the body mass of the ambulatory control rats was consistently higher than that of the HLU group. However, no significant reduction in body mass was observed in the HLU group between day 7 after unloading and time of dissection (data not shown). No significant changes in blood volume were identified between groups (data not

Body mass during experimental period. Male rats initially

Timeline of unloading events

Timeline of wounding and harvesting of tissue

FIG. 1. Timeline of unloading, wounding, and harvesting of tissue. Rats selected for the hindlimb unloading (HLU) or control groups were placed in suspension cages and allowed to acclimate to the cages for 3 days (day −3 to day 0). On day 0, HLU rats were subjected to unloading using a tail suspension apparatus, while control rats remained in suspension cages. HLU rats were allowed to acclimate to the unloading for 14 days (days 0–14). HLU rats were removed from the unloading apparatus on day 14 for wounding but were resuspended immediately after recovery and remained suspended for the duration of the study. Rats were wounded at day 14 after acclimation to unloading. Day 3, day 7, day 10, day 14, and day 21 wounds, blood, and right and left soleus, gastrocnemius, and tibialis anterior muscles were collected during dissection at the indicated time points.

Rats from control or HLU rats

Rats from control or HLU rats

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shown). However, at each time point the weight of the soleus and gastrocnemius muscles from HLU rats was significantly less than that of control rats, confirming the efficacy of the HLU model indicated by hindlimb muscle atrophy (data not shown) (39).

Hindlimb unloading delays wound closure. To determine if simulated bed rest influenced wound reepithelialization, we examined epithelial migration across the wound bed. At days 2 and 3 postwounding, wound sections obtained from hindlimb exposed rats (Fig. 3B) were significantly less reepithelialized compared with those of ambulatory controls (Fig. 3A). Arrows indicate the distance migrated by the keratinocytes across the wound bed. Normal skin is seen on either side of the wound, indicated by dark pink staining. However, by day 7 there was no significant difference in wound coverage between the two treatment groups, with wounds from both treatment groups completely reepithelialized by day 7 (Fig. 3C).

Hindlimb unloading inhibits wound angiogenesis. Since we observed a delay in wound closure by keratinocytes, we postulated that this may also prompt alterations in endothelial cell function. Thus we next sought to determine if mechanical unloading would impair wound angiogenesis. Photographs of tissue sections of wounds from hindlimb exposed rats exhibited fewer vessels compared with wounds from ambulatory controls. Vasculature from wounds of control rats appears to migrate toward the epithelium (Fig. 4A). In contrast, vasculature from wounds of HLU rats is more punctate and lacks directional migration toward the epithelium (Fig. 4B). Analysis of wound vascularity (Fig. 4C) revealed significantly lower wound vascularity at day 7 postwounding; however, no significant difference was observed at days 10, 14, or 21 postwounding. Although the percent vascularity was reduced in wounds from HLU rats, the pattern of angiogenesis was normal, with peak vascularity at day 7 and vascularity equivalent to uninjured skin by day 21.

Hindlimb unloading does not induce changes in proangiogenic cytokines during wound healing. To further assess the effect of hindlimb unloading on reduced wound angiogenesis, we examined the levels of two key proangiogenic cytokines in wounds. To ensure that any alterations in the levels of FGF-2 and VEGF would not be neglected, we chose to assess the levels of these cytokines at time points directly before and after their normal peak to include days 3, 7, and 10 postwounding (22, 45). No significant differences in the levels of FGF-2 (Fig. 5A) or VEGF (Fig. 5B) were observed between control or HLU rats.

Hindlimb unloading does not affect wound hydroxyproline content. After observing a significant reduction in wound vascularity, despite similar levels of proangiogenic cytokines, we next determined if this may have been due to a reduction in wound collagen levels. To determine the level of collagen content in the wound, we quantified the total levels of collagen in wounds from ambulatory or HLU-treated rats using hydroxyproline as an index for total collagen content (Fig. 6). Hydroxyproline content was not significantly altered between either group at any time point following wounding. The pattern of hydroxyproline content, indicative of the amount of collagen being deposited in the wound bed, follows that of a normal healing wound, with levels of hydroxyproline significantly below that of normal, uninjured skin in day 3 wounds and
increases above normal, uninjured skin by day 7 in both controls and HLU rats (45). In addition, total hydroxyproline content in uninjured skin was also not affected by HLU (data not shown).

Differences in wound pathologies induced by HLU. Wounds from days 3, 7, 10, and 14 rats were subjected to histological analysis to detect differences in inflammatory and dermal pathology (Table 1). Wounds from HLU rats had greater neutrophil infiltration within the epidermis and dermis compared with control rats. Furthermore, wounds from HLU rats exhibited a greater population of fibrocytes, or inactive fibroblasts, compared with fibroblasts. However, this did not result in a decrease in total wound collagen content in our studies (Fig. 6), as wounds from HLU rats also exhibited moderate collagen organization within the dermis. Finally, wounds from HLU rats exhibited only mild to minimal lymphocyte and plasma cell infiltrate compared with control rats.

DISCUSSION

The results of this study demonstrate for the first time that previous mechanical unloading promotes global alterations in cutaneous wound healing. Both mechanical loading and physical activity denote bodily movement by skeletal muscles that results in physiological energy expenditure. Although the current belief is that one must undergo vigorous exercise to achieve health benefits, moderate-intensity activities such as walking and resistance training have been shown to considerably improve overall health and healing capability. The recumbent position as part of prolonged bed rest contributes to a reduction in muscular force on bone, energy utilization, and hydrostatic pressure (25). Previous studies have indicated that mechanical unloading dramatically increases the risk for developing secondary complications, including muscle degeneration, cardiovascular dysfunction, thrombosis, diabetes, and chronic wounds (2, 6, 7, 17, 32). At the cellular level, mechanical unloading has been shown to influence gene expression, signal transduction pathways, and proliferative capacity (9, 14, 15, 38). Both exogenous as well as physiological factors contribute to specific cellular alterations associated with abnormal wound healing (37, 53). As additional factors emerge as potential mediators of aberrant tissue repair, the obligation to develop novel interventions designed to improve healing.

Fig. 4. Hindlimb unloading reduces wound vascularity. Histology of day 7 wound sections from ambulatory controls (A) and HLU rats (B). Frozen sections from day 7 to day 21 were stained with anti-PECAM-1 antibody. The area of the wound bed that was used for vascular density analysis is indicated by the dotted line. The percent vascularity for each section is depicted in the bottom left corner. C: percent vascularity (vessel density) was calculated for wounds from HLU (●) or ambulatory controls (○) using image analysis software (Scion Image). Nonselected, normal skin (•). Data are expressed as mean percent vascularity ± SE; n = 6 per group. *P < 0.05 for day 7 by 2-way ANOVA and a Bonferroni posttest.

Fig. 5. Hindlimb unloading does not alter the levels of proangiogenic cytokines in wounds. A: wounds from days 3, 7, and 10 postwounding were collected and homogenized. FGF-2 levels in wound homogenates from hindlimb unloaded (hatched bars) and ambulatory controls (black bars) was determined by ELISA. The peak of FGF-2 expression occurred at day 7 in wounds from both treatment groups. Data are expressed as mean FGF-2 in pg per mg of the average wet weight for each day ± SE; n = 6 per group. Nonselected, normal skin (white bars). P > 0.05 for all time points by 2-way ANOVA and a Bonferroni posttest. Analysis was performed in duplicate. B: wounds from days 7, 10, and 14 postwounding were collected and homogenized. VEGF levels in wounds from HLU (hatched bars) and ambulatory controls (black bars) were determined by ELISA. The peak of VEGF expression occurred at day 7 in wounds from both treatment groups. Data are expressed as mean VEGF in pg per mg of the average wet weight for each day ± SE; n = 6 per group. P > 0.05 for all time points by 2-way ANOVA and a Bonferroni posttest.
becomes progressively more apparent. Despite the clinical evidence that mechanical unloading and prolonged bed rest may be detrimental toward tissue repair, the effects on wound repair using an in vivo model have yet to be fully elucidated. The results from our studies indicate that the normal function of both keratinocytes and endothelial cells is impaired during wound healing in the presence of mechanical unloading.

Nutritional status plays a significant role in the wound healing process, as protein requirements increase to provide the necessary components for protein synthesis. For instance, malnourished patients often present with an increase in wound dehiscence, demonstrating that adequate protein, lipid, and vitamin requirements are met for normal regeneration of the ECM (50). Since we did not observe any significant differences in protein parameters, such as total collagen content or levels of immune cells into the wound for host defense, as well as to provide sufficient oxygenation required for fibroblast proliferation and angiogenesis. Potentially, alterations in fluid shifts that occur during mechanical unloading, such as during spaceflight or prolonged bed rest, may modify normal mechanical forces acting on specific cell populations involved in tissue reepithelialization. However, in these experiments the mice were subjected to control conditions or HLU only 7 days before implantation of corneal sponges, whereas our studies allowed for acclimation to the HLU model for 14 days. Nonetheless, the reepithelialization of corneas in this study was significantly delayed by 6–12 h in hindlimb-suspended mice compared with their ambulatory controls (33). This observation was further assessed, and we found that this impairment of the proliferative phase correlated with a significant reduction in both the rate and magnitude of neutrophil migration into the central regions of the corneal wound. Moreover, the expression of key genes involved in macrophage activity was also significantly reduced in the hindlimb-suspended rats. Since macrophage influx is considered a rate-limiting step during tissue repair, alterations in macrophage influx may have profound effects on various stages of wound healing, particularly the proliferative phase (20, 21, 45). Interestingly, wounds from HLU rats exhibited greater neutrophil infiltration within the epidermis and dermis. Consequently, this may also account for the delay in wound closure, as evidence has suggested that excess inflammation inhibits keratinocyte migration (23). Thus the delay in wound reepithelialization in the present study suggests that the ability of dermal keratinocytes to migrate across the wound bed is impeded by alterations in keratinocyte function. However, these data suggest that the prolonged mechanical unloading delays but does not inhibit keratinocyte migration across the wound bed. Tissue perfusion is critical for both extravasation of immune cells into the wound for host defense, as well as to provide sufficient oxygenation required for fibroblast proliferation and angiogenesis. Potentially, alterations in fluid shifts that occur during mechanical unloading, such as during spaceflight or prolonged bed rest, may modify normal mechanical forces acting on specific cell populations involved in tissue

Table 1. Differences in wound pathologies observed between control or HLU rats

<table>
<thead>
<tr>
<th>Factor</th>
<th>Wound</th>
<th>Control</th>
<th>HLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermal</td>
<td>Day 3</td>
<td>Minimal to mild neutrophil and macrophage infiltrate</td>
<td>Mild to moderate neutrophil infiltrate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate to mild lymphocyte and plasma cell infiltrate</td>
<td>Minimal to mild lymphocyte and plasma cell infiltrate</td>
</tr>
<tr>
<td>Epidermal</td>
<td>Day 7</td>
<td>Mild lymphocyte and plasma cell infiltrate</td>
<td>Mild lymphocyte and plasma cell infiltrate; Severe neutrophil infiltrate</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Panniculus carnosus</td>
<td>Day 3</td>
<td>Minimal or immature collagen present; fibroblasts and myofibroblasts present</td>
<td>Mostly immature myofibroblasts present; minimal collagen formation</td>
</tr>
<tr>
<td>Superficial dermis</td>
<td>Day 7</td>
<td>Fibroblasts present; minimal collagen organization</td>
<td>Fibrocytes present; moderate collagen organization</td>
</tr>
<tr>
<td>Dermis</td>
<td>Day 7</td>
<td>Fibroblasts present; minimal collagen organization</td>
<td>Fibrocytes present; moderate collagen organization</td>
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Histological assessment of wound pathology in wounds from control or hindlimb-unloaded (HLU) rats. Data represent arbitrary differences in inflammation (epithelial and dermal) or extracellular matrix (fibroblast population and collagen organization). Only differences between the two groups are indicated.
repair. If so, then the advantage of mechanical loading may be more dynamic in that it provides both a fluid and physical component to the regulation of cellular function. During unloading, there is a cephalic fluid shift and exclusion of the head-to-toe hydrostatic pressure gradient that promotes orthostatic intolerance, altered tissue perfusion, reduced aerobic capacity, and decreased peripheral vascular resistance (16, 28, 46, 58). Patients subjected to a 6° head-down tilt for 14 days showed decreased cutaneous vasodilator capacity, which was preserved if the subject participated in daily aerobic exercise training during the study (18). Furthermore, rats subjected to HLU exhibited significant hypotension and bradycardia, enhanced vasoconstriction, and vascular resistance (35, 58), indicating that variations in orthostatic homeostasis have the potential to elicit systemic alterations that can impair the wound-healing process.

During tissue repair, keratinocytes secrete various factors that participate in the signaling of endothelial cells in an effort to promote revascularization of the wound bed. Both FGF-2 and VEGF are potent inducers of both physiological and pathological angiogenesis, with VEGF being the most critical cytokine for wound angiogenesis (10, 22, 29, 45). Although the reduction in VEGF was not statistically significant at day 3 ($P = 0.12$), there was a trend toward a decrease in wound VEGF levels, which may be sufficient enough to delay the angiogenic signal for initiation of angiogenesis. The reduction of angiogenesis despite near-normal levels of proangiogenic cytokines in the present study suggests that mechanical unloading may also have a direct effect on endothelial cell function. Endothelial cells are highly sensitive to changes in biomechanical homeostasis, as they function as regulators of blood flow, immune cell adhesion, coagulation cascade, and vascular smooth muscle cell growth (1, 42, 54). Because angiogenesis is such a tightly controlled mechanism, minute changes in hemodynamics or mechanical forces may antagonize endothelial cell proliferation, a critical element of physiological angiogenesis during wound healing. Furthermore, recent studies have suggested that mechanical unloading considerably impacts endothelial cell function. Monocyte adhesion to endothelial cells was found to be significantly upregulated following HLU, which may impede normal function of endothelial cells and promote an increase in inflammation within the wound bed (31). Moreover, endothelial cells exposed to mechanical unloading were found to downregulate IL-1α, a potent inhibitor of endothelial cell growth, as well as baseline levels of actin, suggesting an adaptation mechanism to restrict angiogenic capacity (12). Concurrently, the lymphatic dysfunction in HLU rats reported by Gashev et al. (28) may further limit the migrational capacity of lymphocytes to the wound bed, thus diminishing the reservoir of growth factors required for normal physiological angiogenesis during wound repair.

VEGF and FGF-2 are both potent inducers of physiological angiogenesis (22). Despite a normal expression pattern of both VEGF and FGF-2 in HLU rats, wound vascularity was still reduced. Nonetheless, wounds from HLU rats demonstrated fewer lymphocytes and plasma cell infiltrate compared with control rats. Since both lymphocytes and plasma cells have been implicated in promoting angiogenesis, perhaps the reduction in lymphocyte and plasma cell numbers within the wound milieu restricts the production of critical factors other than VEGF or FGF-2 required for angiogenesis. Furthermore, microwravity has been implicated in cell survival through alterations in VEGF receptor-2 (VEGFR2) signaling. VEGFR2 has a lower affinity for VEGF compared with VEGF receptor-1 (VEGFR1), but has a direct involvement in endothelial cell signaling, resulting in endothelial cell mitogenesis required for angiogenesis (29). These data in conjunction with the present study suggest a mechanism by which wound angiogenesis is inhibited during mechanical unloading.

Regeneration of the ECM is essential to restore the integrity and elasticity of the injured dermis. As the wound healing process proceeds, regeneration of the extracellular matrix and angiogenesis occur simultaneously, as endothelial cell migration and proliferation employ the ECM as a provisional matrix on which to migrate (13). Rats implanted with polyvinyl acetal sponge discs and subjected to 10 days of spaceflight exhibited a 62% reduction in collagen content compared with those rats subjected to normal gravity conditions (19). For this analysis, the sponge is designed to act as a model of wound healing, as it represents a defined matrix into which growth factors and cells can infiltrate. In these studies, the sponges were infused with pellets designed to release supraphysiological levels of growth factors, FGF-2 and PDGF. Hence, the observed differences may have been apparent because of a more robust fibroblast response mediated by the presence of excess growth factor. In our studies, only physiological levels of FGF-2 were present in the wound, which may explain why no differences were detected. Furthermore, the rats used in our studies were subjected to mechanical unloading 14 days, thus allowing for systemic physiological changes to occur before wounding. Interestingly, rats subjected to HLU demonstrated impairment in the time course of collagen gene expression events during wound repair remodeling of the medial collateral ligament (36). At 7 wk posthealing, the HLU rats exhibited significantly depressed collagen type I, type III, and type V gene expression levels compared with ambulatory control animals, although the levels of hydroxyproline were similar. This suggests that for our results, differences in the genotypic ratios of dermal collagens may contribute to further defects in wound healing that would not be detected by quantitating total hydroxyproline levels in skin wounds. Potentially, prolonged disuse could promote alterations in fibroblast function with respect to appropriate synthesis and temporal expression of specific collagen subtypes during healing.

Limitations to the study. There are limitations to this study that should be addressed in future investigations. While the HLU rats normalized to their conditions, as assessed by stabilization of body mass, the overall reduction in total body mass compared with control rats may be a minor factor that contributed to the delay in wound closure and angiogenesis. Possibly, we may have missed the peak of VEGF production in our analyses, which may have indicated a statistically significant reduction in VEGF before day 3. Furthermore, because the tissue was required to be in a hydrated state for homogenization, there may have been changes in VEGF, FGF-2, or hydroxyproline levels that were not detected due to the normalization of tissues that were different in their initial wet weights.

Future directions. Further studies are required to determine if the delay in keratinocyte migration observed in our studies is indirect due to inflammatory changes within the wound milieu or directly through alterations in keratinocyte signal transduc-
tion. Specifically, it would be of interest to determine whether subpopulations of cells (i.e., macrophages, neutrophils) from HLU animals exhibit a change in localization or phenotype, which may contribute the observed defects in keratinocyte migration and endothelial cell function. Although no significant differences in total collagen content, as measured by hydroxyproline levels, were observed in HLU rats despite a greater amount of immature fibrocytes, further studies are required to determine if differences may exist with respect to wound breaking strength, scarring, the extent of collagen organization, or fibril diameter. In addition, the spatial and temporal expression of collagen subtypes could provide further evidence of perturbations in matrix remodeling during HLU.

**Perspectives**

The data acquired from these studies underline the importance of mechanical loading in the maintenance of normal cellular function during tissue repair. However, at the present time, the mechanism behind the observed suppression of normal keratinocyte and endothelial cell function needs to be further investigated. These data highlight the complexity in attempting to elucidate the underlying mechanisms by which mechanical unloading directly influences tissue repair. Ultimately, these results emphasize the importance of mechanical loading on the healing process and may shed light on specific pathological changes that occur in patients subjected to extended periods of bed rest.

**DISCLAIMER**

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**REFERENCES**


