Temporal extracellular matrix adaptations in ligament during wound healing and hindlimb unloading

D. A. Martinez,1,2 A. C. Vailas,3 R. Vanderby, Jr.,4 and R. E. Grindeland5

1Connective Tissue Physiology Laboratory, Department of Health and Human Performance, and 2Biomedical Engineering Program, Department of Mechanical Engineering, University of Houston, Houston, Texas; 3Office of the President, Idaho State University Administration Building, Pocatello, Idaho; 4Orthopedics Research Laboratory, Department of Orthopedics and Rehabilitation and Department of Biomedical Engineering, University of Wisconsin, Madison, Wisconsin; and 5Life Sciences Research Division, National Aeronautics and Space Administration-Ames Research Center, Moffett Field, California

Submitted 15 June 2007; accepted in final form 9 August 2007

Martinez DA, Vailas AC, Vanderby R Jr, Grindeland RE. Temporal extracellular matrix adaptations in ligament during wound healing and hindlimb unloading. Am J Physiol Regul Integr Comp Physiol 293: R1552–R1560, 2007. First published August 15, 2007; doi:10.1152/ajpregu.00423.2007.—Previous data from spaceflight studies indicate that injured muscle and bone heal slowly and abnormally compared with ground controls, strongly suggesting that ligaments or tendons may not repair optimally as well. Thus the objective of this study was to investigate the biochemical and molecular gene expression of the collagen extracellular matrix in response to medial collateral ligament (MCL) injury repair in hindlimb unloaded (HLU) rodents. Male rats were assigned to 3- and 7-wk treatment groups with three subgroups each: sham control, ambulatory healing (Amb-healing), and HLU-healing groups. Amb- and HLU-healing animals underwent bilateral surgical transection of their MCLs, whereas control animals were subjected to sham surgeries. All surgeries were performed under isoflurane anesthesia. After 3 wk or 7 wk of HLU, rats were euthanized and MCLs were surgically isolated and prepared for molecular or biochemical analyses. Hydroxyproline concentration and hydroxylsylpyridinoline collagen cross-link contents were measured by HPLC and showed a substantial decrement in surgical groups. MCL tissue cellularity, quantified by DNA content, remained significantly elevated in all HLU-healing groups vs. Amb-healing groups. MCL gene expression of collagen type I, collagen type III, collagen type V, fibronectin, decorin, biglycan, lysyl oxidase, matrix metalloproteinase-2, and tissue inhibitor of matrix metalloproteinase-1, measured by real-time quantitative PCR, demonstrated differential expression in the HLU-healing groups compared with Amb-healing groups at both the 3- and 7-wk time points. Together, these data suggest that HLU affects dense fibrous connective tissue wound healing and confirms previous morphological and biomechanical data that HLU inhibits the ligament repair processes.

collagen; cross-links; hindlimb suspension; real-time quantitative PCR; rat

THE LIKELIHOOD OF AN ASTRONAUT or cosmonaut receiving an injury that would require repair is highly probable on extended duration spaceflight missions, such as during duty on the International Space Station or on travel to the Moon or Mars. Some astronauts and cosmonauts participate in extravehicular activity, by moving, manipulating, and constructing high-mass hardware and/or participate in other tasks that may increase the probability of being injured. Unfortunately, there is very little spaceflight data from human, nonhuman primate, or rodent experiments on injury repair or trauma associated with the musculoskeletal system. The few pieces of data obtained from studies on rats have indicated that weightlessness may retard the repair of load-bearing bone and skeletal muscle during acute spaceflight exposure (20, 21, 24). Wound repair in the extracellular matrix (ECM) of skeletal muscle has also been shown to be delayed after 14 days of spaceflight (36). Rodent cutaneous tissues subjected to wound repair during spaceflight also showed an impaired ability to heal and then respond to in vivo platelet-derived growth factor (PDGF-BB) and bovine basic fibroblast growth factor (bFGF) supplantations (11).

A common way to simulate the effects of spaceflight on the musculoskeletal system has been with the implementation of the rat hindlimb unloading (HLU) model, used by National Aeronautics and Space Administration (NASA) and investigators worldwide, to study the physiological adaptations associated with acute and chronic microgravity (28, 29). Muscular atrophy and decreased muscle strength are common observations in rats subjected to acute bouts of HLU (3, 17, 33, 35, 37). Our laboratory (35, 38–40, 42) has previously demonstrated that bone and dense fibrous connective tissues subjected to acute bouts (7–28 days) of HLU are significantly compromised in strength, integrity, and biochemical composition because of a reduction in ground reaction forces (GRF). Rodents subjected to 14 and 21 days of HLU had significantly weaker bone-ligament-bone junctions (40) and reduced Achilles tendon ultimate stress and tangential moduli (1). In essence, HLU has deleterious effects on the strength and energy storage capacity of tissues containing ECM collagens.

Ligament tissues are injured more often than any other joint structure (2), and ligament injuries result in a significant disability in ~100,000 patients per year. Despite current modalities of treatment, 15% of the patients with a medial collateral ligament (MCL) injury develop early signs of arthritis, suggesting an incomplete return to knee stability. Because knee ligaments have a high incidence of injury on Earth, the possibility exists that weakening of the knee structure and surrounding tissues during space travel could predispose astronauts and cosmonauts to potential ligament injuries and probable reloading injuries on return to the Moon, Mars, or Earth after extended-length stays in space.

Obtaining access to subjects that have orthopedic wounds and subjecting them to space travel is virtually impossible...
because of numerous factors. We have created a ground-based rodent model to study the orthopedic repair of ligaments during reduced GRFs. The biomechanical, morphological, and histological properties of transected rat MCLs subjected to 21 and 49 days of HLU were previously studied by our research group (30). HLU time course healing experiments revealed significant reductions in ligament maximal force, ultimate stress, elastic modulus, and low-load properties that did not improve ligament strength and stiffness to presurgical status over time (30). HLU animals possessed MCLs with abnormally formed scars and uneven fibroblast cell distribution compared with ambulatory ground controls, suggesting that GRFs are important in the micro-organization of the collagen fibrillar network at the ligament scar site (30). More recently, HLU was also shown to inhibit cell proliferation of the corneal epithelium in a mouse model to study wound healing in the eye (25). HLU caused a delay in the onset of corneal wound closure by ~12 h over a 96-h period (25).

The primary purpose of this study was to elucidate the biochemical and molecular gene expression changes in the healing MCL challenged by acute (3 wk) and extended-duration (7 wk) HLU. We hypothesized that HLU would: 1) diminish the healing capabilities of surgically transected knee ligaments to self-repair, 2) compromise the biochemical properties of the ligament ECM, and 3) alter the ECM gene expression profiles of the healing MCLs. The healing characteristics of the rodent MCL tissues was assayed with specific biochemical and gene markers to assess the integrity, maturation, and molecular organization of collagen, the main ECM protein located within ligaments. Biochemical analyses were conducted of 1) hydroxyproline (Hyp), an amino acid marker used as an index of total collagen content measured by HPLC, 2) the mature collagen cross-link hydroxylysylpyridinoline (HP), which improves ECM strength and tissue quality in healing ligaments, also measured by HPLC, and 3) MCL cellularity by DNA measurement to assess the state of cell proliferation in the healing ligament. Real-time quantitative PCR gene markers were used to determine the upregulation or downregulation of specific genes that impact the ECM in healing MCLs as previously determined (15, 18, 19, 32, 34). To assess new collagen synthesis in remodeling ligament, the genes encoding collagen type I (Col1A2), collagen type III (Col3A1), and collagen type V (Col5A1) were assayed. In addition, genes encoding other ECM ligament constituents important to MCL tissue function were measured: lysyl oxidase, the enzyme catalyzing collagen cross-link formation; decorin and biglycan proteoglycans, which are important in the assembly of collagen fibrils during fibrillogenesis; fibronectin, an ECM structural protein expressed during early wound healing; matrix metalloproteinase-2 (MMP-2 or gelatinase-A), a degradative enzyme that aids in the resorption and remodeling of the ECM; and finally tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), which inhibits the activity of specific MMPs during remodeling. Together, the quantitative measurement of the genes mentioned will provide information on the molecular ECM profile of healing MCLs during HLU.

MATERIALS AND METHODS

Experimental Design

The Institutional Animal Use and Care Committees at the University of Houston, University of Wisconsin-Madison, and NASA-Ames Research Center approved this study. Specific details of the animal model and design of the experiment have been published previously by our research group (30). Male Sprague-Dawley rats (200 g; Simonsen Labs, Gilroy, CA) arrived at NASA-Ames Research Center and were allowed to acclimatize in cages for 7–10 days. Two groups of 30 animals each were divided into 3- and 7-wk wound-healing groups. Each time group contained 10 randomly selected animals that were divided into the following subgroups: sham control, ambulatory healing (Amb-healing), and HLU-healing.

Rats in the Amb-healing and HLU-healing groups were subjected to bilateral MCL transection under isoflurane inhalation anesthesia using a non-rebreathing delivery system via nose cone. All animal surgeries were performed in aseptic conditions in a sterile field. Bilateral skin incisions of ~6–8 mm in length were made on the medial aspect of the knee, exposing the MCL at the femoral-tibial joint line. The MCL transection was made with a no. 11 sterile scalpel blade. The wound area was immediately sutured closed with simple interrupted surgeons’ knots using 4-0 chronic gut (Ethicon, Somerville, NJ) in the subcutaneous fascia and 3-0 Ethilon black monofilament nylon to close the skin. The wound area was cleaned, and Panalog antibiotic ointment was applied postoperatively as needed. Sham control animals were prepared and surgically manipulated in the same manner; however, no surgical transection was performed on the MCL. Postsurgery analgesic (3 μl Tylenol-codeine (120 mg/5 ml Acetaminophen, 12 mg/5 ml codeine phosphate) was delivered to each animal’s 500-ml water bottle for 72 h. Sham and Amb-healing animals were free to move about in their cages after surgery. Animals subjected to HLU were allowed 24 h of recovery before being suspended.

HLU rats were deprived of GRF to their hindlimbs with the use of the Morey-Holton tail suspension protocol at NASA-Ames Research Center Animal Care Facility as previously published (29). Animals were maintained in a 24°C room, exposed to a 12:12-h light-dark cycle, and given rat chow and water ad libitum. Research staff and veterinarians checked animals two or three times per day for skin wound health, overall well being, water (ad libitum) and food consumption (Purina rat chow), and adaptation to the HLU harness on their tails. The nylon sutures were removed 5 days postoperatively with no apparent stress to the animals. The animals were acclimatized after either 3 or 7 wk of unloading. There were 20 MCLs per group, which were distributed and stored at ~85°C as follows: 10 ligaments per group were for protein (biochemical) analyses and 10 ligaments per group were submersed in 200 μl of RNALater (Ambion, Austin, TX).

Biochemistry

Tissue sample preparation. For preparation of tissues for DNA measurement, the method of Vailas et al. (41) was used. An individual lyophilized MCL (1.7–3.6 mg) was placed into brown borosilicate glass screw vials with polytetrafluoroethylene-silicone-lined screw caps. Each vial received 1.2 ml of a papain (P-3125; Sigma-Aldrich, St. Louis, MO) solution (1 ml = 28 mg of protein, 33 U of enzyme/mg protein; therefore, 924 U/ml of papain enzyme were used to digest ligament samples). This solution contained 0.001 M cysteine-hydrochloride, 0.01 M EDTA, and 200 μl/10 ml digest of papain suspension (type III; Sigma-Aldrich) in a 0.01 M phosphate buffer (pH 6.5). In addition, the DNA standards (Sigma-Aldrich) were subjected to the same treatment to determine recoveries for DNA. Samples were heated in an oven at 65°C for 48 h. Digests were extracted with chloroform in a chloroform-to-digest volume ratio of 10:1 and centrifuged at 5,000 rpm for 10–15 min. Samples were frozen at ~20°C until analyzed. The recoveries for DNA were between 94% and 98% for each assay as reported using similar methodology (9).

Contralateral MCL tissues destined for imino acid (Hyp) and trifunctional peptide (HP cross-link) analyses were hydrolyzed in 1.0 ml of 6 M HCl for 24 h at 110°C using brown borosilicate polytet-
ruffluoroethylene-silicone-lined screw-top vials further sealed with Teflon tape. Individual aliquots (200 μl for Hyp and 700 μl for HP cross-links) of each MCL sample hydrolyzate were aliquoted to 12 × 75-mm borosilicate test tubes, evaporated to dryness (Savant Speedvac Thermo-Fisher, Holbrook, NY), and stored at −80°C until further analyses.

**Hyp analyses.** Hyp is a specific index for all phenotypes of collagen, representing 14% of collagen’s mass. Amino acid analyses of Hyp was performed with the precolumn derivatizing Edman reagent phenylisothiocyanate (Pierce Chemicals, Rockford, IL) before quantification with a known amount of derivatized Hyp (Sigma-Aldrich) standard, and the recovery for the imino acid analyses was 95%.

**HP collagen cross-link content determination.** The collagen cross-link analysis (HP) was performed by ion-paired C18 reverse-phase HPLC (Waters 2695 Alliance) method, previously used by our laboratory (26). Aliquots (700 μl) of the dried, hydrolyzed samples were redissolved in 0.1 ml of sample diluent (5 mM sodium phosphate dibasic, pH 7.40 in 5% acetonitrile by volume), filtered through a 0.45-μm filter (GHP Acro-Prep filter plate, Pall Life Sciences, Ann Arbor, MI), eluted isocratically (140 mM sodium acetate-tetraethylammonium-EDTA buffer solution) at a 1.0 ml/min flow rate using a 10 cm × 0.6 mm C18 PICO-TAG column (Waters, Milford, MA) on a Waters model 2695 Alliance HPLC, and monitored on a Waters model 2487 dual wavelength UV/Vis detector at 254 nm. The retention time for Hyp was 2.6 min, the imino acid chromatograms were quantified relative to a known amount of derivatized Hyp (Sigma-Aldrich) standard, and the recovery for the imino acid analyses was 95%.

**DNA content measurement.** The estimation of the ligament cell density was achieved by determining the amount of DNA using a modified method of Bonting and Jones (5) and as previously per-formed by our laboratory (26). Three aliquots (700 μl of original dried hydrolyzate) were redissolved in 1.0 ml of sample diluent (5 mM sodium phosphate dibasic, pH 7.40 in 5% acetonitrile by volume), filtered through a 0.45-μm filter (GHP Acro-Prep filter plate, Pall Life Sciences, Ann Arbor, MI), eluted isocratically (140 mM sodium acetate-tetraethylammonium-EDTA buffer solution) at a 1.0 ml/min flow rate using a 10 cm × 0.6 mm C18 PICO-TAG column (Waters, Milford, MA) on a Waters model 2695 Alliance HPLC, and monitored on a Waters model 2487 dual wavelength UV/Vis detector at 254 nm. The retention time for Hyp was 2.6 min, the imino acid chromatograms were quantified relative to a known amount of derivatized Hyp (Sigma-Aldrich) standard, and the recovery for the imino acid analyses was 95%.

**Molecular Biology.**

**RNA extraction.** Frozen MCLs were placed into RNase-free 1.5-ml round bottom microcentrifuge tubes (Eppendorf-Brinkman) containing a 4.7-mm stainless steel ball. The isolation of total RNA from ligament was performed with Mixer Mill 300 (Qiagen, Valencia, CA) with two 24-well adaptors and the RNeasy dense fibrous tissue isolation kit (Qiagen) according to the manufacturer’s protocol. Total RNA was quantitated by small dilutions and measured by 260-nm optical density on a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE).

**CDNA synthesis.** Complimentary DNA was synthesized by running 30-μl reverse transcription reactions using SuperScript II RNase H− reverse transcriptase (Invitrogen, Carlsbad, CA) according to the company’s instructions with some minor modifications by our laboratory. In the first denaturation reaction, 200 ng of ligament total RNA was added to 1.5 μl of oligo dT(15) primer and diethyl pyrocarbonate water to a total volume of 20.1 μl. Each sample was incubated at 70°C for 10 min in an MJ thermocycler (MJ Research/Bio-Rad, Hercules, CA). After denaturation, a second reaction solution was added containing 6.0 μl of 5× first-strand buffer, 3.0 μl of 0.1 M DTT, 0.6 μl of 200 ng/μl dNTPs, and 0.3 μl of SuperScript II Moloney murine leukemia virus RNase H− reverse transcriptase (50 U/μl) for a total of 9.9 μl per tube. Samples were incubated at 42°C for 50 min followed by 70°C for 15 min. The cDNA samples were stored at −20°C until further quantitative PCR processing.

**Real-time quantitative PCR analysis.** For each gene of interest, 5′ Taqman hydrolysis quantitative PCR assays were performed by an MX-4000 real-time quantitative PCR machine (Stratagene, La Jolla, CA). For each quantitative PCR assay, the sequences for forward and reverse oligonucleotide primers (20–24 nt), fluorescent reporter probes (20–25 nt) containing a 5′-FAM, -HEX, or -Cy5 fluorescent reporter, and a 3′-BHQ 1–3 quencher (Biosource International, Camarillo, CA) were generated with Beacon design software (Premier Biosoft, Palo Alto, CA). The genes of interest and sequences are listed in Table 1. Standards were generated with synthetic DNA amplicon oligonucleotides (Biosource International) spanning the entire quantitative PCR amplification (75–150 nt) region. A standard curve methodology was performed to quantitate the specific gene targets of interest. Specific standard aliquots (100 ng to 10^4 copies of CDNA) were used to generate a linear curve (R^2 = 0.995) with a quantitative PCR efficiency (slope) of −3.3 to determine the cDNA copy number of triplicate replicates. Each quantitative PCR reaction consisted of 12 min at 95°C to activate the HotStar Taq polymerase (Qiagen) followed by 40 cycles of two-step thermocycling (45 s at 94°C and 12 s at 60°C). The CDNA copy number of the target gene was normalized to the copy number of cyclophilin A, which remained constant independent of treatment protocol. Quantitative PCR gene expression measurements were converted to mean multiples of changes compared with 3-wk sham animals. Multiples of changes from 0 to 1 and 0 to −1 are nonlinear. Therefore, the

### Table 1. Real-time quantitative PCR primer and probe sequence data and corresponding accession numbers

<table>
<thead>
<tr>
<th>Gene Markers</th>
<th>Forward Primer</th>
<th>Taqman Probe</th>
<th>Reverse Primer</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col 1A2</td>
<td>CAGAACATCACCTACACCTGCAAG</td>
<td>CTCTCTGTCGAGCTCAGGAGATG</td>
<td>TGGCAAGATCGAGCTTATCCAG</td>
<td>AF121217</td>
</tr>
<tr>
<td>Col 3A1</td>
<td>CATGCTCGAGAGGGGGGATG</td>
<td>TGGGGGCGGGTTCCTCACCATATAGG</td>
<td>TGGTTGCTGTCGAGCTTATCCAG</td>
<td>X70369</td>
</tr>
<tr>
<td>Col 5A1</td>
<td>GCTGACGAGCTGAGGGGGGATG</td>
<td>CAAGCGCGACGAGCTGAGCTTACGG</td>
<td>TTTTCTGAGCTGAGCTTATCCAG</td>
<td>AE273662</td>
</tr>
<tr>
<td>MMP-2</td>
<td>CCAAGGAGCATGGTCGAGGAGGCTG</td>
<td>AGACACGAGCTCAGTGGACGAACG</td>
<td>TGGCTGAGCTGAGCTTATCCAG</td>
<td>M19533</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>GAGGAGGCTGACGAGGAGGAGGCTG</td>
<td>AGGCTGAGCTGAGCTTATCCAG</td>
<td>TGCAAGATCGAGCTTATCCAG</td>
<td>J02903</td>
</tr>
</tbody>
</table>

MMP-2, matrix metalloproteinase-2; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

**AJP-Regul Integr Comp Physiol • VOL 293 • OCTOBER 2007 • www.ajpregu.org**
multiples of changes of mean gene expression were log$_{10}$ transformed before statistical analysis was performed.

**Statistical Analysis**

To evaluate the differences among groups for each dependent variable, a two-way ANOVA was applied. When the F tests were significant, Duncan’s post hoc tests and least square means used to further identify differences between group pairs. Statistical significance level was set at $P < 0.05$.

**RESULTS**

**Body Weights**

All animals tolerated the anesthesia, MCL surgical transection, recovery, and HLU well. The initial body weights were not significantly different at the beginning of the time course study (Table 2). As with the majority of rodent HLU protocols, the HLU animals failed to grow at the rate of the sham and Amb-healing groups within the first 7 days, as previously shown in rat growth charts from an earlier publication by our research group (30). HLU-healing body weights at the end of each study were significantly smaller than age-matched sham and Amb-healing groups ($P < 0.05$).

**Extracellular Matrix Biochemistry**

There was a significant (15–25%) decrease ($P < 0.05$) in Hyp concentration (Fig. 1), an index of total collagen, in both the 3-wk Amb-healing and HLU-healing groups compared with the 3-wk sham group. All 7-wk groups showed recovery to sham control levels. The HP collagen cross-link content (Fig. 2), a physiological marker of collagen maturation, was significantly (25–30%) smaller ($P < 0.05$) in the 3- and 7-wk Amb- and HLU-healing groups. The content of HP cross-links was significantly greater ($P < 0.05$) in the 7-wk sham group than in the 3-wk group. HLU and wound repair in the MCL did not affect the accumulation of HP cross-links compared with Amb-healing in either the 3- or 7-wk groups.

**Tissue Cellularity**

We used tissue DNA concentration to measure tissue cellularity. The DNA concentrations (Fig. 3) in the 7-wk HLU-healing group was significantly greater ($P < 0.05$) than in the 3-wk sham control group and all other groups. The tissue cellularity was smaller (30–60%) in 7-wk sham and Amb-healing groups. The tissue cellularity in the 7-wk HLU-healing group remained significantly elevated compared with that shown in sham and Amb-healing age-matched controls ($P < 0.05$).

![Fig. 1. Hydroxyproline (Hyp) concentration within the surgically transected medial collateral ligament (MCL) in ambulatory (Amb), hindlimb unloaded (HLU), and sham groups allowed to heal 3 and 7 wk. Values, expressed as Hyp concentration (µg of Hyp/dry wt tissue), are means ± SE. All transected ligament groups, in addition to the 7-wk sham control group, were compared with the 3-wk sham control group. Means with the same letter are not significantly different from each other ($P > 0.05$). *Hyp concentration in the 3-wk Amb and HLU groups are significantly smaller than that shown in the 3-wk sham group ($P < 0.05$).](http://ajpregu.physiology.org/)

**MCL Tissue Gene Expression Levels**

Changes in collagen types I, III, and V gene expression are shown in Fig. 4, and the specific numerical values and changes (in fold) of other ECM markers are shown in Table 3. The HLU-healing group demonstrated impairment in the time course of collagen gene expression events during wound repair remodeling compared with that shown in the Amb-healing group. In Fig. 4A, collagen type I (Col1a2) gene expression levels remained constant during the first 3 wk of wound healing. However, at 7 wk of wound healing, the HLU-healing

![Fig. 2. Hydroxylysylpyridinoline (HP) collagen cross-link content within the surgically transected medial collateral ligament (MCL) in ambulatory (Amb), hindlimb unloaded (HLU), and sham groups allowed to heal 3 and 7 wk. Values, expressed as HP content (mol HP/mol of collagen), are means ± SE. All transected ligament groups, in addition to the 7-wk sham control group, are compared with the 3-wk sham control group. Means with the same letter are not significantly different from each other ($P > 0.05$). **HP cross-link contents in the 3-wk Amb and HLU groups are significantly smaller than results in the 3-wk sham group ($P < 0.05$). *7-wk sham group HP cross-link content is significantly larger than that shown in all other groups ($P < 0.05$).](http://ajpregu.physiology.org/)
levels are virtually absent in the 7 wk Amb-healing group. Similarly, biglycan, a small cell surface or pericellular proteoglycan with similar properties to decorin, shows similar decrements at 3 wk in the HLU-healing group and remains significantly elevated compared with the 7 wk Amb-healing group (P < 0.05). Results for lysyl oxidase, the gene that codes for the enzyme that catalyzes collagen cross-link formation, are

Fig. 3. Tissue cellularity is measured by DNA tissue concentration within the surgically transected MCL in Amb, HLU, and sham groups allowed to heal 3 and 7 wk. Values, expressed as DNA concentration (μg DNA/dry wt tissue), are means ± SE. All transected ligament groups, in addition to the 7-wk sham control group, are compared with the 3-wk sham control group. Means with different letters are significantly different from each other (P < 0.05). **Tissue cellularity in the 3-wk HLU group is significantly larger than that shown in all other groups (P < 0.05). Tissue cellularity results in the 7-wk sham (**) and Amb (**) groups are significantly smaller than those shown in all other groups (P < 0.05).

Fig. 4. Real-time quantitative PCR gene expression measurements of collagen type I (A; Col1a2), collagen type III (B; Col3a1), and collagen type V (C; Col5a1) phenotypes within the surgically transected MCL in Amb, HLU, and sham groups allowed to heal 3 and 7 wk. Gene expression values, expressed as fold changes, are means ± SE. All transected ligament groups, in addition to the 7-wk sham control group, are compared with the 3-wk sham control group. Means with the same letters are not significantly different from each other (P > 0.05). In A, gene expression of Col1a2 in the 7-wk HLU group is significantly smaller than that shown in all other groups (**P < 0.05). In B, gene expression of Col3a1 in the 7-wk Amb group is significantly larger than that shown in all other groups (**P < 0.05). In C, gene expression of Col5a1 in the 7-wk HLU group is significantly lower than that shown in all other groups (**P < 0.05).
also shown in Table 3. Lysyl oxidase is reduced in the 3-wk Amb-healing and HLU-healing groups (P < 0.10), which parallels the collagen cross-link content in Fig. 2. However, HLU-healing does not alter the expression of lysyl oxidase vs. the Amb-healing controls. As shown in Table 3, MMP-2 (gelatinase A) and TIMP-1 were normally elevated during normal and pathological ECM remodeling. MMP-2 activity was altered in the HLU-healing group at 7 wk compared with the Amb-healing group (P < 0.05). No changes in MMP-2 gene expression from sham controls were apparent at 3 wk (Table 3). TIMP-1 expression at 3 wk was significantly reduced in the (3 wk) HLU-healing group (P < 0.05) compared with the Amb-healing group; however, TIMP-1 expression remained greater in the HLU-healing group at 7 wk.

### DISCUSSION

Data from this study support our hypotheses that HLU diminishes the healing of dense fibrous connective tissues and degrades the repair processes. This is evidenced by changes in collagen ECM, changes in tissue cellularity, and altered gene expression of ECM components involved in ligament wound repair processes. The biochemical and molecular analyses confirm the biomechanical measurements from our previous study showing significantly reduced maximal force, ultimate stress, and elastic modulus in HLU-healing animals after 3 wk of MCL healing, which was not improved with an additional 4 wk of ligament healing (30). HLU-healing animals at 3 and 7 wk also showed altered cellular and ECM microarchitectures at the scar site compared with results shown in Amb-healing animals, suggesting that GRFs are necessary for proper collagen fiber alignment and orientation (30).

Extracellular protein and molecular gene markers characterizing healing ligaments in rats, rabbits, and humans have been well documented in reviews on injury and repair of ligaments and tendons (13, 14, 18, 31, 44, 45). Results from the Amb-healing groups in our study are consistent with the well-characterized processes of wound healing previously described for the time points used in our study (13, 14). The MCL heals through three processes, including hemorrhage with inflammation, followed by ECM and cellular proliferation, and concluding with active remodeling and maturation of the collagen ECM (14). Evidence provided in this study suggests that genes encoding the three main collagen phenotypes (Col1a2, Col3a1, and Col5a1) within ligaments are differentially expressed in HLU-healing animals compared with Amb-healing animals at 3 and 7 wk, thus emphasizing the importance of mechanical stress on ligament ECM gene regulation during wound healing.

### ECM Collagen and Maturation

A significant reduction in the imino acid Hyp, a marker of total collagen in connective tissues, in both of the 3-wk surgically wounded groups (Amb-healing and HLU-healing) is in agreement with rat and rabbit time-course wound healing models of ligaments and tendons (6, 16, 31). Postsurgical tendon gap transection in rats showed little change in Hyp after 30 days (6). However, tendon ultimate stress increased to 70% of controls and the HP collagen cross-link doubled in content, suggesting that an improvement in tendon strength following wound healing can occur without an increase in total collagen (6). Similarly, Achilles tendon repair in rabbits was studied 15 days after surgery and showed that healing tendons recovered 80% of the collagen and 60% of the HP collagen cross-links compared with normal tendons (31). The mature HP cross-links have also been reported to never obtain presurgical levels 1 yr postinjury after subsequent surgical repair (18), which may, in part, contribute to the repaired tissue’s decrement in postsurgery strength. In our study, a decrement in total collagen and HP cross-link contents after MCL surgery was demonstrated in both surgical wound healing groups. Furthermore, HLU did not improve or reduce collagen concentration or maturation compared with amputating animals. An additional 4 wk of healing showed no differences in collagen content or maturation vs. sham controls, suggesting that other ECM factors, such as the organization of the collagen fibers or proportion of specific collagen phenotypes comprising the ECM, may contribute to our previously reported strength decrements in both the 3- and 7-wk groups (30).

### Tissue Cellularity

Ligament repair during early wound healing is well characterized by a disorganized, hypercellular repair area at the scar site with a large number of inflammatory cells and migrating fibroblasts (14, 23). Matrix flaws and a greater degree of cellularity are indicative of a delay in the wound repair process (14, 23). In our study, ligament cellularity was measured via DNA content from papain-digested sham and surgically transected ligaments. HLU-healing animals had significantly elevated levels of cellularity 3 wk postsurgery, and the levels remained elevated at 7 wk postsurgery. The tissue cellularity

### Table 3. Medial collateral ligament real-time quantitative PCR gene expression changes compared with 3-wk sham control group

<table>
<thead>
<tr>
<th>Gene Marker</th>
<th>3 Weeks</th>
<th>7 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amb-Healing</td>
<td>HLU-Healing</td>
</tr>
<tr>
<td></td>
<td>Sham Control</td>
<td>Amb-Healing</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>2.58</td>
<td>2.57</td>
</tr>
<tr>
<td>Decorin</td>
<td>1.67</td>
<td>0.35**</td>
</tr>
<tr>
<td>Biglycan</td>
<td>1.45</td>
<td>0.58</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>0.57</td>
<td>0.47†</td>
</tr>
<tr>
<td>MMP-2</td>
<td>1.28</td>
<td>1.21</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.27</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Values are expressed as mean fold change compared with the 3-wk sham control group, which was set at 1.0 (data not shown). *Significantly different from the 3-wk sham control group (P < 0.05). Means with different letters are significantly different from each other (P < 0.05). †Lysyl oxidase for 3-wk HLU-healing compared with the 3-wk sham control group (P < 0.10).
Differential Expression Extracellular Matrix Genes During Ligament Repair

New molecular methodology for dense fibrous connective tissues, which contain a substantially reduced number of fibroblasts compared with other tissues, has allowed researchers to measure the differential expression of key genes involved in the ligament repair process from attomole quantities of reverse-transcribed total RNA. Early studies investigating gene quantitation and ligament repair in rabbits by Hart and colleagues (8, 32, 34) have provided seminal data on the differential expression of genes encoding structural proteins and enzymes during wound repair. On the basis of previous literature (32, 34), key regulatory genes (collagens type I, III, V, fibronectin, lysyl oxidase, decorin, biglycan, MMP-2, and TIMP-1), involved in the production and remodeling of the ligament ECM were measured from sham and surgically repaired MCLs. Supporting our hypothesis that HLU delays ligament wound healing, the expressions of collagen types I (Col1a2), III (Col3a1), and V (Col5a1) in the 7-wk HLU-healing group were significantly reduced compared with that shown in Amb-healing animals. Interestingly, there was no significant difference in collagen gene expression at the 3-wk time point. The reduced gene expression of collagen types I, III, and V at the 7-wk time point in the HLU-healing group could indicate that the expression of newly synthesized collagen may be reduced during HLU even though the total collagen (new + old collagen protein) showed no change compared with the Amb-healing groups. The expression of type III collagen is usually greater than the expression of type I collagen during early wound healing. However, with time, further tissue remodeling proceeds and type I collagen becomes the predominant fibrillar collagen while simultaneously improving dense fibrous connective tissue strength (14). Our results indicate that HLU differentially suppresses collagen types I, III, and V expression at 7 wk, thereby influencing the remodeling phase of ligament wound healing.

In addition, fibronectin, a key protein that aids in the synthesis of provisional granulation tissue during early wound repair, is expressed at greater levels in the 7-wk HLU-healing group. Similarly, decorin and biglycan, proteoglycans involved in the purported inhibition and/or regulation of collagen fibril and fibrin assembly (10, 12, 43), demonstrated a significantly greater expression in the 7-wk HLU-group compared with the Amb-healing group, suggesting that normal fibrillogenesis in the HLU-healing animals may be compromised. The importance of decorin gene and protein expression in ligament healing needs further study.

MMP-2 (gelatinase A) and TIMP-1 gene expression levels were measured to determine whether the collagen remodeling process during wound healing and HLU was altered at different time points. MMP-2 activity is well known to digest basement membrane (type IV collagen) and type I collagen more efficiently than other collagenases (7, 22). TIMP-1 inhibits collagen degradation by inhibiting MMP activity, and its greater level of expression during wound healing in ligament suggests that TIMP-1 may regulate collagen resorption by MMPs. In our study, a smaller expression of MMP-2 and a greater TIMP-1 expression level in the 7-wk HLU-healing group suggest that ECM turnover and remodeling may be inhibited by HLU. It is unknown whether HLU has a direct effect on normal MMP activity during tissue remodeling. Future studies dedicated to measuring the delicate balance of MMP and TIMP enzyme activity through zymography in dense fibrous connective tissue during the combination of wound healing and unloading could confirm protein turnover and remodeling activity. Finally, the gene encoding lysyl oxidase, a copper-dependent metalloenzyme that catalyzes the first steps in collagen cross-link formation (reducible cross-link formation), showed a trend toward less expression at 3 wk in the HLU-healing group. HP cross-link formation is depressed in both of the 3-wk surgical groups; however, it remains uncertain whether labile reducible collagen cross-link formation confers the same strength within the healing tissue compared with mature stable collagen cross-links and/or whether there is a direct correlation between lysyl oxidase gene expression and collagen cross-link formation during wound healing.

Limitations

This study has limitations that should be addressed in future investigations. Immunohistochemical localization of proteins in and near the scar area would help in ascertaining the protein expression of the specific genes that were measured using real-time quantitative PCR. In addition, the use of in vivo collagen formation markers, such as the conversion of \(^3\)H]proline to \(^3\)H]Hyp to measure new collagen secretion (specific activity), as well as gel zymography to determine ECM resorption, could provide further evidence of potential dense fibrous connective tissue turnover rates during wound healing and HLU.

HLU is a model that mimics some of the physiological aspects of spaceflight; in our experiments, the model was used to decrease the GRFs during ligament wound healing to simulate potential orthopedic healing situations that may occur during long-term space travel. Using the HLU model, other investigators have suggested that wound healing is also depressed in other tissues with an adequate blood supply, such as in corneas (25). We postulate that a decreased blood flow to the hindlimbs could exacerbate the wound healing process during HLU as previously demonstrated in rat HLU studies measuring vascular changes (4, 27).

In summary, our data support the concept that HLU depresses normal ligament wound healing processes and produces inferior ECM material at the wound transection site.
Biomechanical, biochemical, and molecular gene expression data show a diminished wound healing response of the MCL when deprived of GRFs.

ACKNOWLEDGMENTS

The authors thank the following individuals at the NASA-Ames Research Center who contributed to animal care: Tom Wang, Fang Yuan, Megan Moran, Lisa Baer, Cyra Fung, Guilia Gurun, and David Garcia. We also express appreciation to the members of the Connective Tissue Physiology Laboratory who helped in the preparation of the rat MCL biochemical and gene expression assays: Dr. Antonios Kyparos, Dr. Laura Gutiérrez, Kavita Davé-Coombs, Andrea Creath, Nancy Garcia, John Fink, Hai-Yen Hoang, Phuong Le, and Andrew Lust.

GRANTS

This research was supported by National Aeronautics and Space Administration Grant NAG9-1152 (D. A. Martinez, A. C. Vailas, R. Vanderby, and R. E. Grindeland).

REFERENCES