Exposure-Dependent Increases in IL-1β, Substance P, CTGF, and Tendinosis in Flexor Digitorum Tendons with Upper Extremity Repetitive Strain Injury

Jane M. Fedorczyk,[1] Ann E. Barr,[2] Shobha Rani,[3] Helen G. Gao,[3] Mamta Amin,[4] Shreya Amin,[4] Judith Litvin,[3] Mary F. Barbe[2,3,4]

[1]Department of Physical Therapy and Rehabilitation Sciences, Drexel University, 245 North 15th Street, MS502, Philadelphia, Pennsylvania 19102, [2]Department of Physical Therapy, Thomas Jefferson University, 130 South 9th Street, Philadelphia, Pennsylvania 19107, [3]Department of Anatomy and Cell Biology, Temple University Medical School, 3400 North Broad Street, Philadelphia, Pennsylvania 19140, [4]Department of Physical Therapy, Temple University, 3307 North Broad Street, Philadelphia, Pennsylvania 19140

Received 18 February 2009; accepted 17 July 2009
Published online 9 September 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20984

ABSTRACT: Upper extremity tendinopathies are associated with performance of forceful repetitive tasks. We used our rat model of repetitive strain injury to study changes induced in forelimb flexor digitorum tendons. Rats were trained to perform a high repetition high force (HRHF) handle-pulling task (12 reaches/min at 60 ± 5% maximum pulling force [MPF]), or a low repetition negligible force (LRNF) reaching and food retrieval task (three reaches/min at 5 ± 5% MPF), for 2 h/day in 30 min sessions, 3 days/week for 3–12 weeks. Forelimb grip strength was tested. Flexor digitorum tendons were examined at midtendon at the level of the carpal tunnel for interleukin (IL)-1β, neutrophil, and macrophage influx, Substance P, connective tissue growth factor (CTGF), and periostin-like factor (PLF) immunoexpression, and histopathological changes. In HRHF rats, grip strength progressively decreased, while IL-1β levels progressively increased in the flexor digitorum peritendon (para- and epitendon combined) and endotendon with task performance. Macrophage invasion was evident in week 6 and 12 HRHF peritendon but not endotendon. Also in HRHF rats, Substance P immunoexpression increased in week 12 peritendon as did CTGF- and PLF-immunopositive fibroblasts, the increased fibroblasts contributing greatly to peritendon thickening. Endotendon collagen disorganization was evident in week 12 HRHF tendons. LRNF tendons did not differ from controls, even at 12 weeks. Thus, we observed exposure-dependent changes in flexor digitorum tendons within the carpal tunnel, including increased inflammation, nociceptor-related neuropeptide immunoexpression, and fibrotic histopathology, changes associated with grip strength decline. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:298–307, 2010

Keywords: cytokines; inflammation; flexor digitorum tendon; repetitive task; PLF; CTGF; WMSD

Tendinopathies of the hand and wrist tendons are associated with forceful repetition in the workplace.[1–3] The incidence of flexor tenosynovitis is significantly higher in strenuous meat processing jobs: 25.3% for female packers, 16.8% for female sausage makers, and 12.5% for male meat cutters. The incidence in non-strenuous jobs was less than 1% during a 31 month study period.[4,5] Manufacturing workers performing highly repetitive and forceful jobs are 29 times more likely to develop wrist and hand tendonitis than workers performing low repetition and low force jobs.[5,6]

The etiology and pathophysiology of overuse-induced tendinopathies are still under investigation. Although the presence of an inflammatory component has not been identified by all investigators,[7–9] increased inflammatory molecules, for example, PGE_2, have been found in tenosynovium of patients diagnosed with carpal tunnel syndrome (CTS), especially during the intermediate phase.[10,11] However, PGE_2 was not found in tendon biopsies collected during the chronic painful tendinosis stage, although increased glutamate neurotransmitter and its receptor were evident.[8,9] The neurochemical Substance P is associated with chronic pain mediation[12] and has also been identified in tendons of patients with chronic tendinopathies.[13–15] Long-term tendinopathies are also characterized by tenosynovial hyperpalgesia, tenocyte rounding, and endotendon disorganization.[16–18]

We have developed a rat model of voluntary repetitive reaching and grasping in which reach rate and force can be varied in order to investigate the pathophysiology of repetitive motion injuries of the upper limb.[19–24] After 3–12 weeks of a high repetition, negligible force task (HRNF; eight reaches/min at 5 ± 5% maximum pulling force [MPF]) for 2 h/day, 3 days/week, animals show decline in motor performance, and increased macrophages and cytokines in flexor digitorum tendons at wrist level and surrounding tenosynovium.[19,20] These changes are exposure-dependent, with a low repetition, negligible force (LRNF) exposure having a greatly diminished response.[20,23] When high force (60% MPF) is added to high repetition (HRHF), increased median nerve fibrosis (increased connective tissue growth factor [CTGF] immunopositive fibroblasts and collagen type I deposition in epineurial tissues in the carpal tunnel region) with concomitant reduced nerve conduction velocity are observed,[22] as are skeletal degenerative changes in distal radius and ulna bones.[24] However, we have yet to examine exposure-dependent degenerative changes in tendons in our model or the inflammatory response in tendons with performance of a HRHF task.

We hypothesize that inflammation begins earlier than fibrotic and other degenerative tendon changes with performance of repetitive upper extremity tasks, and that these responses are exposure dependent (with greater tissue responses over time and with higher demand tasks). We also hypothesize that inflammatory

Correspondence to: Mary F. Barbe (T: 215-707-4896; F: 215-707-7500.; E-mail: mary.barbe@temple.edu)

and neurochemical tendon changes are related to decline in grip strength, a behavior test that correlates with movement-related hyperalgesia.[25,26] Therefore, in this study, we examined grip strength, IL-1β production and immunoexpression, influx of inflammatory cells, Substance P immunoexpression, and signs of tendon histopathology, including increased expression of two matricellular proteins produced by fibroblasts, CTGF and PLF, in flexor digitorum tendons at the level of the carpal tunnel in rats exposed to either a low demand or a high demand voluntary repetitive reaching task.

METHODS

All experiments were approved by the Temple University Institutional Animal Care and Use Committee in compliance with NIH guidelines for the humane care and use of laboratory animals. Studies were conducted on female Sprague-Dawley rats (285–310 g; Ace, PA), housed in a central animal facility in separate cages on a 12 h light:dark cycle with free access to water.

Subjects

Eighty-seven female, young adult Sprague-Dawley rats were used, which were 12–14 weeks of age at the onset of task training. Thirty-two rats were trained to perform a HRHF forelimb handle-pulling task for a food reward for 3 ($n=4$), 6 ($n=9$), or 12 weeks ($n=19$) as described previously.[22,24] Seventeen rats were trained to perform a LRNF forelimb food pellet retrieval task for 3 ($n=3$), 6 ($n=4$), or 12 weeks ($n=10$) as described previously.[23] Both tasks were performed for 2 h/day, in 30 min sessions, separated by 1.5 h breaks, 3 days/week for 3, 6, or 12 weeks. Twenty-eight rats were age-matched normal controls. Ten more were age- and weight-matched trained control rats that were food restricted and went through the initial task shaping period, but did not proceed to the task regimen with the experimental task rats (see ref. 20). The task and trained rats were food-restricted and maintained within ±5% of the body weight of age-matched controls. Rats were allowed to use their preferred limb to reach, termed the "preferred reach" limb (Fig. 1B,C). The contralateral limb in the HRHF rats was used for postural support, termed the "postural support" limb (see Fig. 1B).

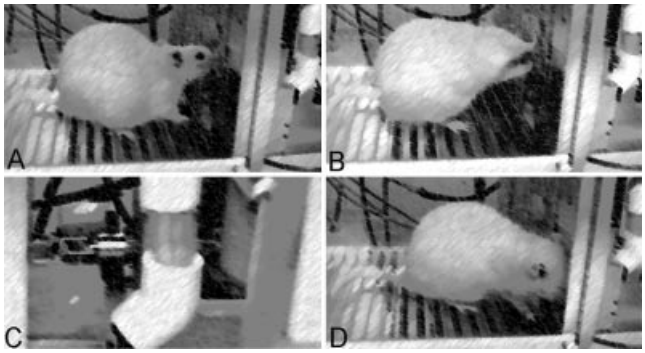

Figure 1. Rat performing HRHF repetitive reaching task. (A) Rat awaits auditory stimulus with snout in portal. (B) Rat reaches for force handle with left forepaw; right forepaw used for postural support. (C) Viewed from top, rat grasps and isometrically pulls force handle attached to force transducer, until predetermined force threshold is reached and held for at least 50 ms. (D) Rat retrieves foot pellet reward by mouth from food trough.

Determination of Grip Strength

Maximum forearm grip strength was determined bilaterally for all control and HRHF rats weekly. LRNF grip strength data were previously reported.[23] Briefly, rats were lifted gently by the tail and allowed to grasp a rigid bar attached to a force transducer and digital display unit (Stoelting, Wood Dale, IL), as described previously.[22,23] When the first signs of active grasp were observed, the rats were pulled upward slowly by the tail with increasing firmness until their grasp was overcome. The peak force was recorded as maximum grip strength. The test was repeated three to five times/limb, and maximum grip strength per trial included in the statistical analysis.

Measurement of Tendon IL-1β by ELISA

Rats were euthanized with sodium pentobarbital (120 mg/kg body weight). The flexor digitorum tendons were collected from rats performing the HRHF task for 6 ($n=5$) or 12 ($n=5$) weeks, and from normal controls ($n=10$), at 18 h after last task performance. The flexor digitorum tendons were separated from the muscle belly using a scalpel (in rats the flexor digitorum muscle is not clearly divided into superficial and profundus heads; therefore, we examined the combined flexor digitorum tendons) (see Fig. 3K,L), lumbrical muscles removed, then tendons rinsed in sterile saline, homogenized, and lysates analyzed for IL-1β using ELISA as described previously.[20] Each sample was run in duplicate and data (pg cytokine protein) normalized to microgram total protein.

Immunohistochemical Analyses

Immunohistochemical analysis was performed on flexor digitorum tendons collected from normal controls ($n=12$), trained controls ($n=9$), and rats that had performed either the HRHF task for 3 ($n=4$), 6 ($n=4$), or 12 weeks ($n=4$), or the LRNF task for 3 ($n=3$), 6 ($n=4$), or 12 weeks ($n=3$). Animals were euthanized, perfused transcardially with 4% paraformaldehyde in PO_4 buffer, and forearm musculotendinous tissues were dissected as a mass off the forearm bones (as shown in Fig. 3K,L) and sectioned longitudinally as a soft tissue mass (en bloc) as described previously.[19,23] Sections, on slides, were incubated in 3% H_2O_2 in methanol (4°C) for 30 min, washed, incubated in 4% dried milk in PBS (Blotto) for 20 min, and then overnight at room temperature with a Substance P antibody (#MAB1566; Chemicon, Temecula, CA; 1:500 dilution with 4% Carnation milk in PBS). After washing, sections were incubated for 2 h at room temperature with goat antimouse peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. HRP was visualized as a black immunoreactive stain using diaminobenzidene (DAB) with cobalt (Sigma-Aldrich, St. Louis, MO). For IL-1β and periostin-like factor (PLF; labels activated fibroblasts producing this matricellular protein), sections were immunolabeled and detected with HRP-DAB as previously described.[21,24] Eosin and/or nuclear red were used as counterstains. A series of adjacent sections were also stained with hematoxylin and eosin (H&E) only. Sections were dehydrated and coverslipped with DPX mounting medium. For CTGF (a fibroblast growth factor that induces collagen production and an activated fibroblast marker), sections were immunolabeled and detected with Cy3 (red fluorescence), and coverslipped with 80% glycerol in PBS, as previously described.[22] Negative control slides included omission of either the primary antibody or the secondary antibody.

Selected sections were double-labeled after Substance P immunolabeling with either anti-ED1 (detects a 90 kDa lysosomal membrane protein in monocytes/macrophages) or anti-PGP9.5 (a pan neuronal marker). After Substance P immunolabeling with secondary antibody conjugated to Cy2 (green tag; Jackson ImmunoResearch; diluted 1:100 in PBS for 2 h), tissue sections were washed, digested with 0.5% pepsin in 0.01 N HCl for 20 min at room temperature, and then incubated with goat serum (4%) in PBS for 30 min at room temperature. Sections were then incubated with either anti-ED1 (MAB1435; Chemicon; 1:250 dilution in 4% goat serum in PBS) or anti-PGP9.5 (ab8189; Abcam, Cambridge, MA; 1:50 dilution in 10% goat serum in PBS) overnight at room temperature. Sections were incubated with appropriate secondary antibodies conjugated to Cy3 (red tag; Jackson ImmunoResearch). Slides were coverslipped with 80% glycerol in PBS. Selected sections were also double-labeled with CTGF and collagen type I antibodies as described previously.[22]

Quantitative Analyses of Histological and Immunohistochemical Findings

To determine the changes in IL-1β and Substance P in flexor digitorum tendons, HRP-DAB stained slides were quantified by two naïve examiners, using a microscope (Nikon E800) interfaced with a digital camera and a bioquantification software system (Bioquant Osteo II; Bioquant, Nashville, TN). Prior to acquisition, the camera was white-balanced and the microscope's light intensity and camera gain maintained at a constant level to ensure similar background values for each acquired image. Separate measurements of the paratendon, epitendon, and endotendon regions of the mid-tendon (carpal tunnel) region of the flexor digitorum tendons were made using the irregular region of interest (ROI) option for the Bioquant software using a 40X objective and a thresholded area fraction quantification method as described previously.[21,23] This determination was made at the level of the wrist joint (i.e., the carpal tunnel region), and 1 mm proximal and distal to the wrist joint (as shown in Fig. 3K,L), for each tendon, at three microscope field locations per rat in two to three separate sections per rat. Para- and epitendon region measurements were summated to give peritendon measurements because the paratendon and epitendon regions merged with task-induced fibrotic tendon changes. Immunofluorescent CTGF antibody staining was quantified in the same tendon regions using similar methods. To determine the changes in neutrophils and macrophages, H&E stained slides and ED1-immunostained slides, respectively, were examined and the number of each cell type counted per square millimeter in peri- and epitendon using the ROI option of the Bioquant software and a 40X objective.

Assessment of Tendon Histopathology

H&E stained flexor digitorum tendon sections were examined by two naïve examiners for histopathological changes in tendons from normal control rats ($n = 18$), trained control rats ($n = 10$), 12 week HRHF rats ($n = 14$), and 12 week LRNF rats ($n = 10$). The tendon region (shown in Fig. 3K,L) was scored for each forelimb per rat using a modification of a semiquantitative scoring method (Bonar scale) to quantify tendon changes. We scored four factors on a four point (0–3) scale: cell shape, collagen organization, cellularity, and amount of vascularization, in peritendon and endotendon, using the previously described modified Bonar scale.[27,28] Cell shape scores are defined as: 0 (all tenocytes are slender and elongated, i.e., normal morphology); 1 (tendons contain mostly elongated tenocytes, but also a small number of oval cells that are similar in size to normal tenocytes); 2 (equal numbers of elongated tenocytes and rounded cells); and 3 (tendon consists of primarily rounded and enlarged cells. This category can include both rounded tenocytes [i.e., fibroblasts] and macrophages). Collagen fibril organization was examined without polarized light and the scores were defined as: 0 (parallel fibers closely packed together); 1 (slightly wavy but still closely packed); 2 (slightly wavy and separated from each other); and 3 (quite wavy with clear loss of parallel nature and separated from each other). Cellularity scoring ranged from a relative low number of cells to matrix ratio to an abnormally high number of cells. Vascularity, or to be more specific, signs of angiogenesis, in the peritendon was also scored from 0 (no capillary profiles per microscope field viewed with a 20X objective); 1 (<5 capillary profiles); 2 (6–10 capillary profiles); 3 (>10 capillary profiles). The larger median artery also present nearby was not included in this count. Each of these determinations was made at the level of the wrist joint, and 1 mm proximal and distal to that joint (as shown in Fig. 3K,L) for each flexor digitorum tendon at the level of the wrist, at three microscope field locations per rat, in two to three separate sections per rat.

Statistical Analyses

To test for differences in variables between the two control groups (normal vs. trained controls), an unpaired t-test was used (two-tailed). As no statistical differences were observed between the control groups (see Results), all control rats were combined into one group for subsequent comparisons to experimental rats. HRHF rat grip strength and IL-1β ELISA protein levels were analyzed by two-way ANOVA with the factors week and limb (preferred reach and postural support). IL-1β and CTGF immunostaining data, and neutrophil and ED-1-positive macrophage cell count data from the preferred reach limbs were analyzed by two-way ANOVA with the factors region and limb. For Substance P immunostaining data, mixed model, multivariate ANOVAs were used with the factors week, group (HRHF and LRNF), tissue region (peritendon and endotendon), and limb. Data from each microscope field measured (three measurements per region, per limb, and per rat) were used as a blocking factor. A Kruskal-Wallis nonparametric test was used to determine differences in tendon pathology scores. A p value ≤ 0.05 was considered significant. Bonferroni method was used for posthoc analyses, with results compared to controls.

RESULTS

Progressive Decline in Grip Strength with HRHF Task Performance

There were no statistical differences between grip strength in normal control rats versus trained control rats ($p > 0.05$); therefore they were combined into a single control group. Two-way ANOVA examining grip strength changes in HRHF rats showed a difference by week ($p < 0.0001$), but not between limbs ($p = 0.4439$). Posthoc analysis showed that grip strength decreased progressively from weeks 3 to 12, bilaterally, in HRHF rats compared to controls (Fig. 2). Grip strength in the LRNF rats has already been reported to decline transiently in week 6 compared to normal controls, after which (by week 8) it returned to normal levels.[20]

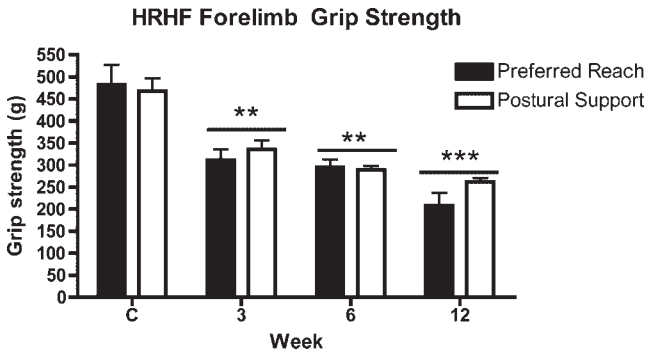

Figure 2. Grip strength significantly decreased in weeks 3, 6, and 12 bilaterally in HRHF rats compared to controls (C; normal and trained control data combined). $^{**}p < 0.01$; $^{***}p < 0.001$ compared to controls.

IL-1β Levels and Macrophages Increase with HRHF Task in Flexor Digitorum Tendons

Few IL-1β immunoreactive cells were present in control flexor digitorum tendons at the level of the wrist (Fig. 3A). IL-1β immunoreactive cells were present in peritendon in 3, 6, and 12 week HRHF flexor digitorum tendons at the level of the wrist (Fig. 3B–D). These results were quantified in the preferred reach limb of HRHF rats and showed a significant difference in percent area of IL-1β immunochemical staining in flexor digitorum tendons at the level of the wrist across weeks of task exposure ($p < 0.0001$), and by region ($p < 0.0001$, peritendon vs. endotendon). Bonferroni posthoc analyses (Fig. 3E) showed that IL-1β was significantly increased in 3 and 6 week HRHF (both $p < 0.05$) and 12 week HRHF peritendon ($p < 0.0001$), and in 12 week HRHF endotendon ($p < 0.01$), compared to controls.

For further confirmation of these results, ELISA was used to examine flexor digitorum tendons bilaterally from an additional cohort of control and HRHF rats. We found significant differences in IL-1β levels by week ($p = 0.0022$), but not by limb ($p = 0.7411$). Posthoc analysis showed that IL-1β levels increased bilaterally (in both preferred reach and postural support limbs) in weeks 6 and 12 HRHF flexor digitorum tendons compared to normal controls (Fig. 3F). We have previously shown that IL-1β does not increase in LRNF flexor tendons compared to normal or trained controls.[20]

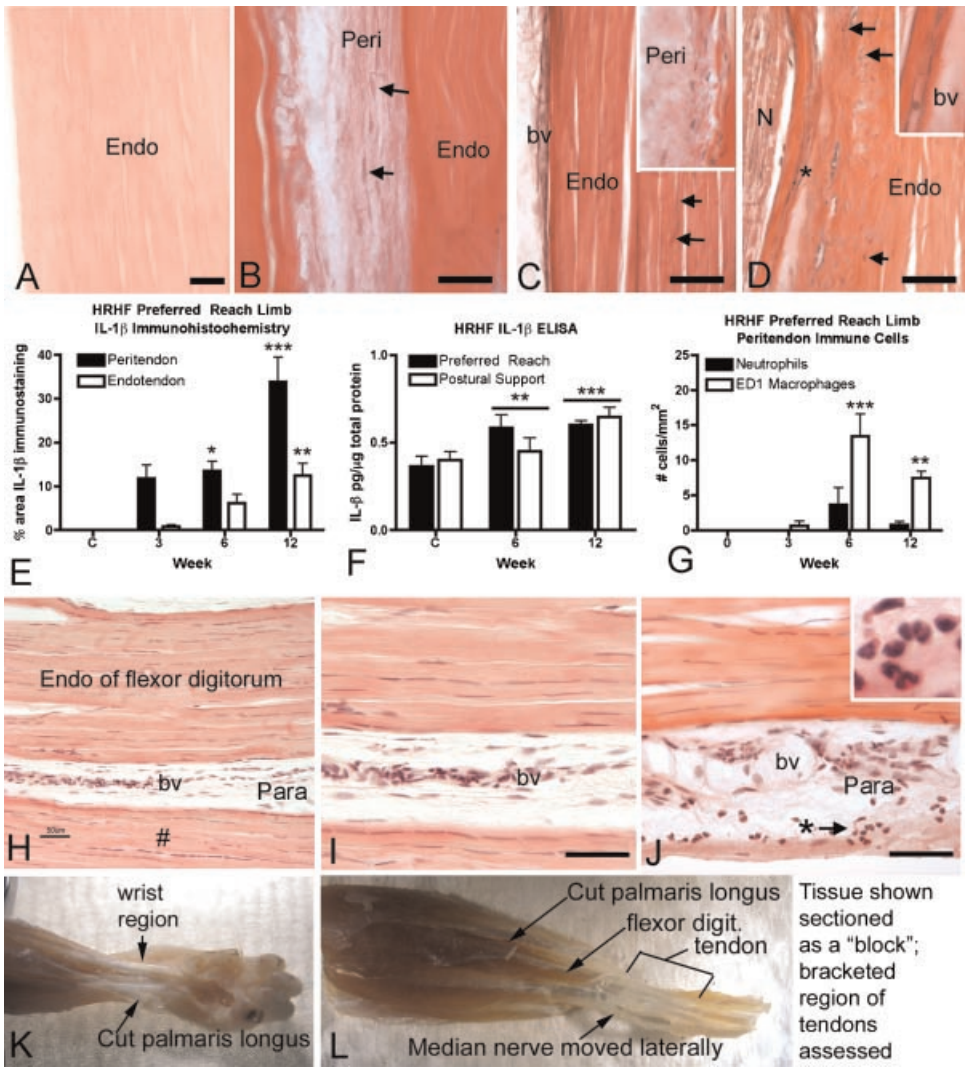

Figure 3. Inflammatory response in longitudinal sections of flexor digitorum tendons at wrist level. (A–D) IL-1β immunoreactive cells in tendons of controls (A), 3 week HRHF (B), 6 week HFHF (inset shows peritendon) (C), and 12 week HRHF (D; inset shows higher power of peritendon blood vessel indicated by an asterisk. Arrows indicate IL-1β stained fibroblasts [tenocytes]). Eosin counterstain. (E) Quantification of IL-1β immunostaining in preferred reach limb's flexor digitorum tendons in peritendon and endotendon from combined normal and trained controls (C) and weeks 3, 6, and 12 HRHF rats. Mean and SEM data are shown. (F) ELISA results of IL-1β levels in preferred reach and postural support flexor digitorum tendons of normal control (C) and week 6 and 12 HRHF rats. Mean and SEM data are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to controls. (H–J) H&E stained tendons showing no neutrophils in control (H,I) but increased neutrophils in 6 week HRHF peritendon (J). Inset in (J) shows higher power of neutrophils indicated by an asterisk. (K,L) 1X macroscope pictures of soft tissue (muscles, tendons, and nerves) from the flexor region of the forelimb before (K) and after dissection (L) from the radius, ulna, and carpal bones. Bracketed region in (L) indicates region of flexor digitorum (digit.) tendon assessed. Scale bars = 50 μm. bv, blood vessel; Endo, endotendon; Peri, peritendon; Para, paratendon region.

In 6 and 12 week HRHF rats, some monocytes in peritendon blood vessels and endotendon tenocytes were IL-1β immunoreactive (Fig. 3C,D and inset in D). ED-1 stained macrophages were counted in the preferred reach limb and showed significant increases in the peritendon at 6 weeks ($p < 0.001$) and 12 weeks ($p < 0.01$) (Fig. 3G), but not in the endotendon (data not shown). We have previously shown that ED-1 macrophages do not increase significantly in LRNF flexor tendons compared to controls.[23] As another indicator of inflammation, we observed H&E stained neutrophils in several 6 week HRHF peritendons (Fig. 3J), cells absent from control peritendon (Fig. 3H,I). Neutrophils were increased at 6 weeks in peritendon, but not significantly compared to controls (Fig. 3G). Neutrophils were not increased in LRNF tendons.

Substance P Increases in HRHF Midtendon Flexor Digitorum Peritendon

There were no statistical differences between Substance P in normal control tendons versus trained control rat flexor digitorum tendons ($p > 0.05$); therefore these data were combined into a single control group. Histologically, the controls and week 3 HRHF flexor digitorum tendons at the level of the wrist showed a clear demarcation between the epitendon and paratendon regions (Fig. 4A,B). However, these boundaries were not clearly differentiated in 12 week HRHF tendons due to hyperplasia-like thickening of these two layers that began by week 3 (see double arrows in Fig. 4B,C). Thus, the paratendon and epitendon measurements were combined for subsequent Substance P data analysis, and are referred to as the peritendon. In 3 and 6 week HRHF, a few rounded Substance P immunoreactive cells were in the peritendon (Fig. 4B; 6 week data not shown). By week 12 HRHF, many small immunopositive cells and elongated profiles were visible throughout the endotendon and peritendon (Fig. 4C,D).

Quantification of immunohistochemistry in flexor digitorum tendons at the level of the wrist showed a significant difference in percent area of Substance P immunoreactivity between groups ($p < 0.0001$), tendon tissue regions ($p = 0.0001$), and weeks of task exposure ($p < 0.0056$), but not between limbs. Bonferroni analyses (Fig. 4E–H) showed that Substance P in 12 week HRHF peritendon significantly increased, bilaterally, compared to controls, and showed a tendency to increase in week 3 ($p = 0.0090$; Fig. 4F). No significant Substance P immunoreactivity was observed in HRHF endotendon or LRNF flexor digitorum tendons (peritendon or endotendon) (Fig. 4E,G, H).

Double-labeling of Substance P immunoreactive cells with PGP9.5, a general neuronal marker, revealed that some Substance P immunostained profiles were axons (Fig. 4D), structures also observed surrounding nearby blood vessels (Fig. 5C). PGP9.5-labeled processes were not observed in control or LRNF rat tendons (data not shown). Substance P immunoreactivity was also observed in 12 week HRHF tenocytes (Fig. 5A), peritendon mast cells (Fig. 5B), and peritendon cells resembling macrophages (Fig. 5D). ED-1 double labeling confirmed that Substance P immunoreactive macrophages were present near blood vessels (Fig. 5E–G).

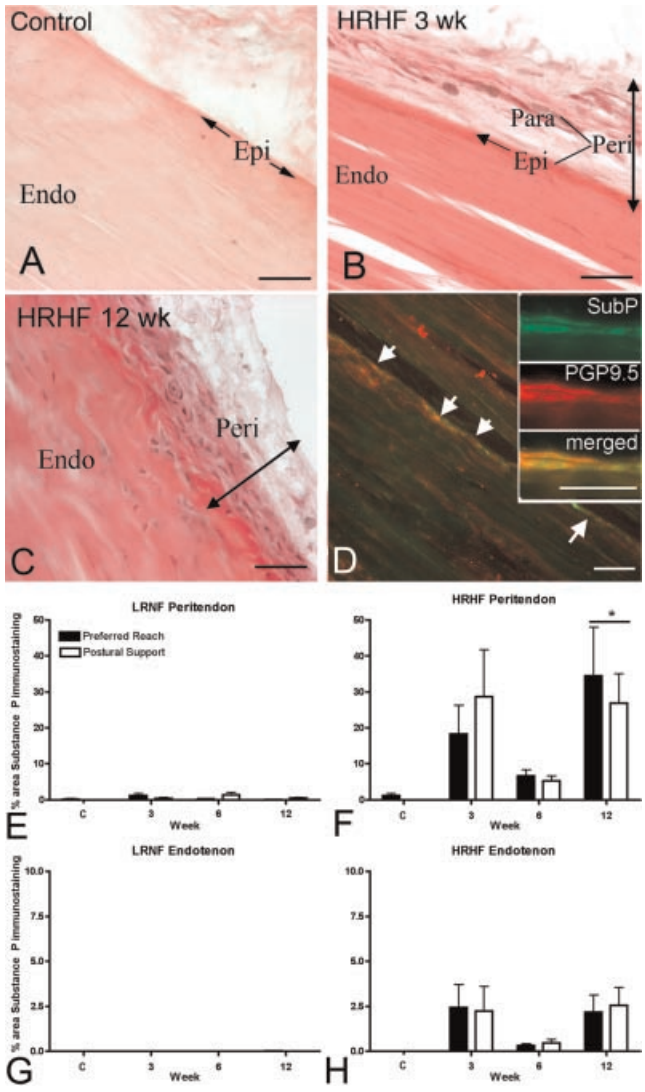

Figure 4. Substance P immunoreactivity in longitudinal sections of flexor digitorum tendons at wrist level. (A–C) Substance P HRP-DAB immunostaining (black) in flexor digitorum tendon sections counterstained with eosin in control (A), 3 week HRHF (B), and 12 week HRHF rats (C). Combined epitendon (Epi) and paratendon (Para), termed the peritendon (Peri), is enlarged in HRHF rats (indicated by double-ended arrow). (D) Substance P (SubP; green) and PGP9.5 (red) double-labeling showing neuronal processes immunoreactive for Substance P in peritendon in 12 week HRHF tendon. Arrows denote double-labeled cells; insets show higher power of processes. (E–H) Graphs showing quantification of Substance P immunoexpression in LRNF and HRHF peritendon and epitendon at weeks 3, 6, and 12 compared to controls (C). Mean and SEM are shown. $^*p < 0.002$ compared to controls. Scale bars = 50 μm. Endo, endotendon.

Progressive Flexor Digitorum Tendon Histopathological Changes

We observed histopathological changes in HRHF 12 week flexor digitorum tendons at the level of the wrist (Figs. 3B–D, 4B,C, 6), but not in LRNF 12 week or control flexor digitorum tendons (Figs. 3A,H,I, 4A,

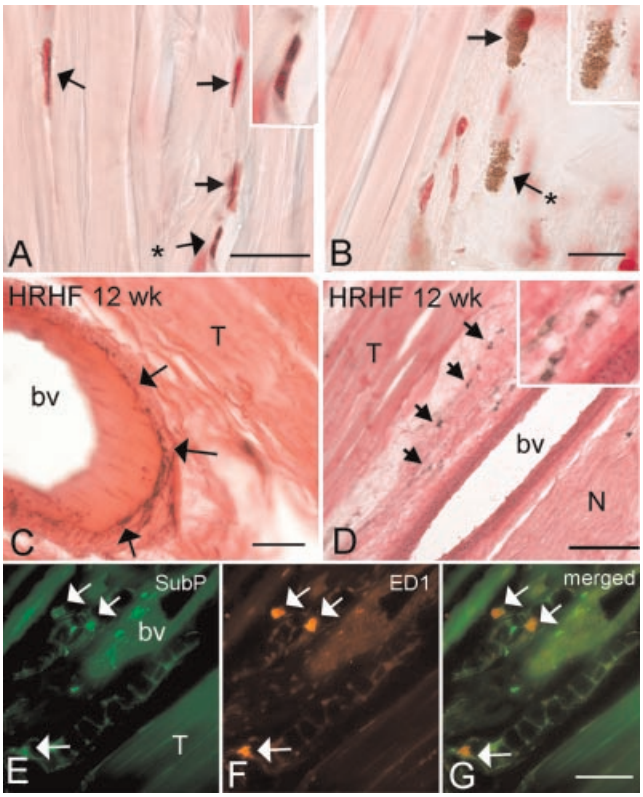

Figure 5. Substance P immunoreactivity in longitudinal sections of flexor digitorum tendons at wrist level. (A,B) Substance P HRP-DAB immunostaining (black) in 12 week HRHF flexor digitorum tendons counterstained with nuclear red showing labeled fibroblasts (A) and mast cells (B). Cells indicated by asterisks are in insets. (C,D) Substance P immunostaining (black) in 12 week HRHF tendons counterstained with eosin showing axonlike processes around blood vessel (C) and macrophage-like cells near blood vessel (D). (E–G) Double-labeling of 12 week HRHF flexor digitorum tendons for Substance P (E; green; SubP) and ED-1 (F; red), and merged (G). Arrows denote double-labeled cells. bv, blood vessel; N, nerve; T, tendon. Scale bars = 50 μm.

6A–C). There was an increase in cellularity, particularly in the peri- or paratendon, in 12 week HRHF flexor digitorum tendons (Fig. 6A left panel, D–G; see also Figs. 3B–D,J, 4C), compared to 12 week LRLF and control tendons (Fig. 6B,C; see also Figs. 3A,H,I, 4A). The increased cells in the peritendon region of HRHF rats included IL-1β-immunoreactive cells (Fig. 3B–D), Substance P immunoreactive cells, axons, mast cells, and macrophages (Figs. 4B–D, 5B,D–G), as well as fibroblasts (Fig. 7). In terms of cell shape changes, control and 12 week LRNF tendons showed slender and elongated tenocytes in the endotendon and thin epitendon (Fig. 6A middle panel, B,C; see also Fig. 3H,I). In contrast, there was an increase in rounded tenocytes observed in the endotendon of 12 week HRHF rats (Fig. 6A middle panel, F; See also Fig. 4C). There was an increase in collagen fiber bundle disorganization (no longer parallel; separations between fibers often visible) in HRHF 12 week flexor digitorum tendons (Fig. 6A right panel, D–G; see also Fig. 4C) compared to 12 week LRLF and control tendons (Fig. 6B,C; see also Fig. 3H,I). The number of small capillary profiles within the peritendon was also scored, but showed only a trend toward an increase with HRHF task performance ($p = 0.07$ compared to controls; graph not shown; Fig. 6E, see also Fig. 7G). Finally, by week 12 in HRHF tendons, the epitendon was often difficult to discern from the paratendon due to epitendon hypertrophy and the spread of cells from the epitendon into the paratendon, thus creating a hyperplagic peritendon (Fig. 6E,G, see also Fig. 4C).

We further explored this peritendon hyperplasia by immunostaining flexor digitorum tendons for CTGF and PLF, both matricellular proteins that increase in fibroblasts that are actively producing collagen type I and PLF into extracellular matrices.[21,24,29] Figure 7C,D shows a clear increase in CTGF-immunoreactive fibroblasts in a thickened peritendon in 12 week HRHF tendons at the level of the wrist. Figure 7D shows CTGF-labeled fibroblasts (red) surrounded by collagen type I immunostaining (green) at a merged peritendon and endotendon interface. Quantification of the preferred reach limb flexor digitorum tendons at the level of the wrist showed significant increases in percent area, with CTGF immunoreactive fibroblasts in HRHF 12 week peritendon compared to control tendons ($p < 0.01$; Fig. 7E). In Figure 7G, we show an increase in PLF in 12 week HRHF peritendon. PLF-expressing fibroblasts were also present within the merged endotendon-peritendon interface (Fig. 7G). A higher power photo (Fig. 7G, inset) shows PLF in the extracellular matrix surrounding these fibroblasts, suggestive of secretion of this extracellular matrix protein into the surrounding tendon matrix by these fibroblasts. We have recently reported using Western blot analyses that PLF increase progressively with task performance in HRHF flexor tendons compared to normal or trained controls.[29]

DISCUSSION

In this study, we found an early decrease (onset by week 3) in grip strength that coincided with the timing of increased Substance P and IL-1β-immunostained cells, and IL-1β production in flexor digitorum tendons at the level of the carpal tunnel in rats exposed to a high demand voluntary repetitive reaching task, but not a low demand task (see also ref. 20). We observed in the HRHF tendons a later increase (12 weeks) in infiltrating macrophages that coincided temporally with the peak in Substance P and tendon histopathology, including increased peritendon cellularity primarily due to increased peritendon CTGF and PLF-immunostained fibroblasts, and increased endotendon collagen disorganization. There was also a trend toward neovascularization by week 12 in HRHF rat tendons.

Forelimb grip strength declined progressively in weeks 3–12, bilaterally, in HRHF animals compared to controls. This extends our previous observations showing decreased grip strength in week 12 in HRHF rats (only week 12 was examined in this earlier study; see ref. 22). The bilateral response was due to the use of each limb in performing the HRHF task, one as the preferred

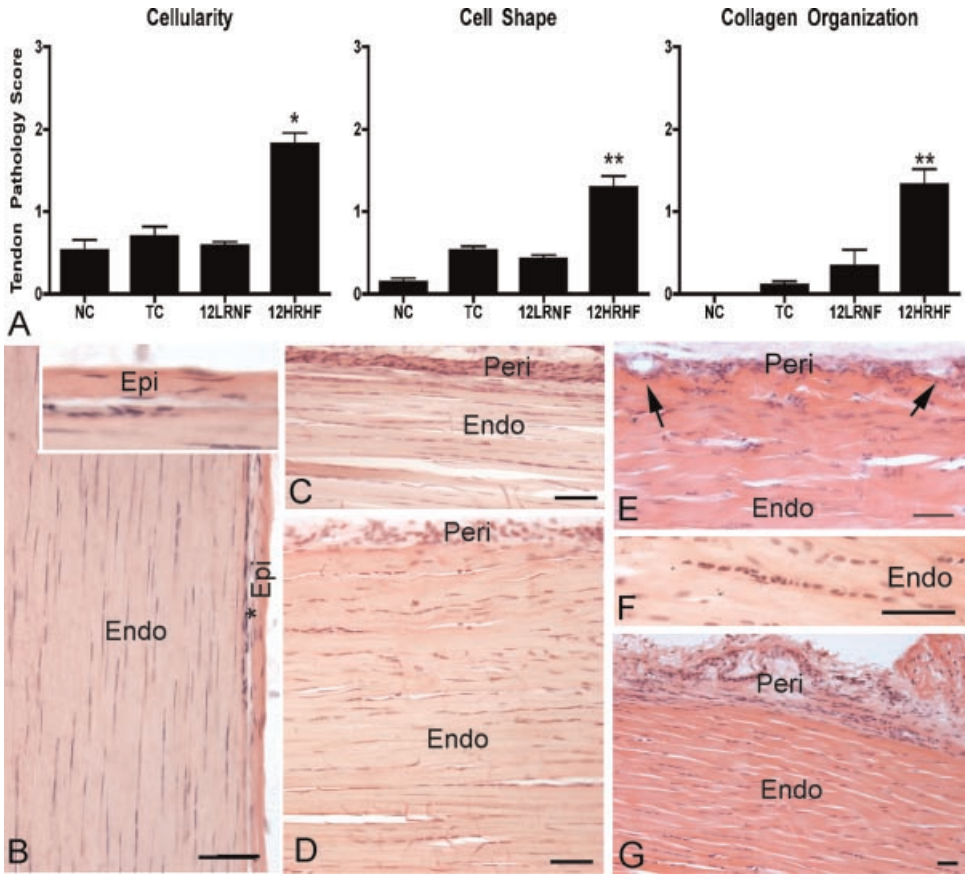

Figure 6. Degenerative change in longitudinal sections of flexor digitorum tendons at wrist level. (A) Tendon pathology scores for cellularity, cell shape, and collagen organization. $*p < 0.05$, $**$ $p < 0.01$ compared to normal controls (NC). Trained controls (TC) also shown. Mean and SEM are shown. (B–F) H&E stained tendons showing control (B and higher power inset of area indicated by asterisk), 12 week LRNF (C), 6 week HRHF (D), and 12 week HRHF (E–G). Endo, endotendon; Epi, epitendon; Peri, peritendon; TC, trained control. Scale bars = 50 μm.

reach limb and the other as the postural support limb. Because decreased forelimb grip strength is an indicator of deep tissue hyperalgesia in animal models of muscle inflammation,[25,26] and the timing of IL-1β increases and grip strength declines are temporally matched, it is likely that tendon inflammation contributed to the grip strength losses. However, Substance P occurrence in tendons is also associated with nociceptive behaviors, such as hind paw thermal sensitivity after Achilles tendon injury.[30] It is likely then that Substance P also contributed to grip strength declines, especially because it showed a trend for an increase at week 3, matching the onset of grip strength decline.

We observed progressive increases in the proinflammatory cytokine IL-1β in HRHF tendons with continued task performance as well as increased inflammatory cells in the peritendon. We have previously found increased cytokines in forelimb flexor tendons of rats performing the HRNF tasks in week 8, but not earlier, and not within tendons of LRNF rats.[20,23] These combined findings support our hypothesis of exposure- dependent tissue and behavioral responses. Increased IL-1β has been shown to be an early response to tendon injury[21,31–33] (levels of mRNA for IL-1β increase only transiently on day 3 in tendon and sheath after a tensile loading injury[31,32]). Increased proinflammatory cytokines (IL-1β and TNFα), neutrophils, and macrophages are observed early after Achilles tendon injury,[21,33] but not in tenosynovial sheaths collected from patients during carpal tunnel surgery, which typically occurs long after the onset of pain (which is most likely the point of injury and associated inflammation) in these patients.[10] However, in patients with lateral epicondylitis, IL-1β-immunoreactive fibroblasts are observed in the extensor carpi radialis brevis muscle origin.[33] IL-1β is known to induce proliferation of fibroblasts.[34] Exposing tendon cells to IL-1β in vitro results in an initiation of tendon matrix destructive pathways, such as matrix metalloproteinase pathways.[35,36] Therefore, our observed early increase in IL-1β most likely contributes to the initiation of the subsequent proliferative and degenerative tendon changes.

Numerous articles have identified Substance P in tendon specimens from patients with painful chronic tendinopathies[7,13,15] and in animal studies of tendon disorders.[14,29] For example, in tendons of patients with chronic medial and lateral epicondylalgia, Substance P immunoreactivity was present in tendon-associated nerve bundles and free nerve endings.[7,13] We also observed Substance P immunoreactivity in free nerve endings in tendon and blood vessel walls, as well as in tenocytes, mast cells, and macrophages, cells identified by others to show Substance P immunoreactivity, especially with tendinosis.[7,13–15,37–39] Likewise, our observed Substance P increases were only in the HRHF task group, a group with histopathological signs of

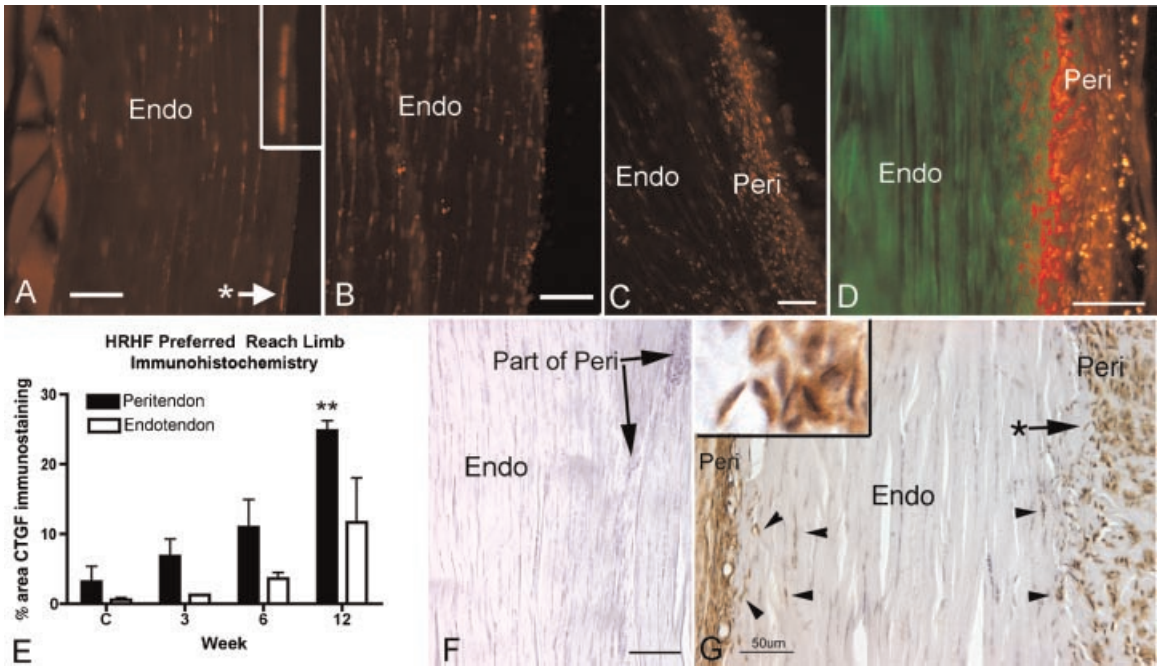

Figure 7. Peritendon fibroblast changes in flexor digitorum tendons at wrist level. (A–D) Connective tissue growth factor (CTGF; red) immunostaining in control (A), 12 week LRNF (B), and 12 week HRHF (C,D) tendons. Inset in (A) shows high power of peritendon area indicated by an asterisk. (D) Double labeling of CTGF (red) and collagen type I (green) in 12 week HRHF tendon. (E) Quantification of CTGF immunostaining in preferred reach limb's flexor digitorum tendons from combined normal and trained controls (C) and weeks 3, 6, and 12 HRHF rats. Mean and SEM data are shown. $^{**}p < 0.01$ compared to controls. (F,G) PLF immunostaining in control (F) and 12 week HRHF (G) tendons. Arrowheads indicate PLF-positive fibroblasts in endotendon. Arrow and asterisk indicate region enlarged in inset, which shows PLF immunostaining in matrix surrounding PLF-stained fibroblasts. Endo, endotendon; Peri, peritendon. Scale bars = 50 μm.

tendinosis. Substance P has several roles, including facilitating histamine release from mast cells and thus enhancing vasodilation and extravasation of immune cells.[40–42] This held true in our model as well, with both Substance P and extrinsic macrophages increasing in week 12 peritendon regions. Because IL-1β facilitates Substance P release,[43] perhaps both contribute to inflammation as well as nociceptive behaviors with tendinopathies. Substance P has also been implicated in the induction of CTGF and proliferation of fibroblasts.[44] This would explain our temporal match in Substance P and peritendon fibrotic tissue changes.

Degenerative changes most commonly observed in overuse tendinopathies include pericellular thickening, fibrosis, and hypercellularity.[11,16,27] Endotendon changes are also commonly observed, and include abnormal tenocyte morphology, increased CTGF immunoreactive cell densities, and collagen fibril disorganization.[16,17,27,28] Our findings are similar to findings by Nakama et al.,[17] although our increase in CTGF-immunoreactive cell densities was most prominent in the peritendon versus within the tendon matrix as in Nakama's study. This difference may be regional, as they examined tendon attachment sites to bone (the enthesis and just distal to it), and we examined unattached flexor tendon regions passing through the wrist region (at least 4–5 mm proximal to the distal enthesis). An examination of the enthesis of flexor carpi radialis tendons and flexor carpal ulnaris tendons in our HRHF rats also showed an increase in CTGF in rounded tenocytes (unpublished data). However, we have chosen to focus on flexor digitorum tendons passing through the carpal tunnel because any thickening of these tendons would likely contribute to a presumed occupation of space by thickened (fibrotic) median nerve epineurium within the carpal tunnel in our model,[22] and therefore to our previously observed declines in conduction velocity in this section of the median nerve.[22] Like inflammation, the tendon histopathological changes in our repetitive strain injury model showed exposure dependence, being absent from LRNF rats, and in HRHF rats.

Our observed increase in CTGF-immunostained fibroblasts in 12 week HRHF flexor digitorum peritendon is highly suggestive of proliferative fibrotic tissue changes in this tendon region. We also observed an increase in CTGF-immunostained fibroblasts at the endotendon and thickening peritendon interface, changes that could be due to a fibroblast invasion of the endotendon. However, the presence of collagen type I around these CTGF-immunostained (and therefore activated) fibroblasts is also suggestive of increased collagen deposition by the more internally located and perhaps more mature fibroblasts. These data match our previous findings of increased CTGF and collagen type I in association with fibrosis-induced compression of the median nerve.[22] We also observed an increase in PLF-immunostained fibroblasts and an increased presence of PLF in the tendon matrix surrounding these fibroblasts. These findings combined with the CTGF and collagen results are suggestive of both peritendon fibroblast

proliferative hyperplasia and matrix deposition with performance of a HRHF task, changes contributing to peritendon thickening and perhaps to some of the endotendon degenerative changes.

In conclusion, our findings support overuse as a factor in tendon injuries. We also found that the development of inflammatory, neuropeptide, and histopathological changes was dependent both on level of task demands as well as on time spent engaged in an overuse activity. The timing of both tendon IL-1β and Substance P increases suggests that they contribute to grip strength declines. Finally, the early increase of tendon IL-1β, a cytokine with roles in initiating fibroblast proliferation and degenerative tendon changes, suggests that this early inflammatory cytokine response is one inducing factor of the later observed fibrotic tendon and degenerative changes.

ACKNOWLEDGMENTS

This work was supported by grants from CDC-NIOSH OH 03970 to M. F. B. and from NIAMS AR051212-01 to A. E. B. No authors have professional or financial affiliations that would bias this work.

REFERENCES

1. Ranney D, Well R, Moore A. 1995. Upper limb musculoskeletal disorders in highly repetitive industries: precise anatomical physical findings. Ergonomics 38:1408–1423.
2. Bystrom S, Hall C, Welander T, et al. 1995. Clinical disorders and pressure-pain threshold of the forearm and hand among automobile assembly line workers. J Hand Surg (Br) 20:782–790.
3. Gerr F, Marcus M, Ensor C, et al. 2002. A prospective study of computer users: I. Study of design and incidence of musculoskeletal symptoms and design. Am J Ind Med 41: 221–235.
4. Kurppa K, Viikari-Juntura E, Kuosma E, et al. 1991. Incidence of tenosynovitis or peritendinitis and epicondylitis in a meat-processing factory. Scand J Work Environ Health 17:32–37.
5. Silverstein BA, Fine LJ, Armstrong TJ. 1986. Hand wrist cumulative trauma disorders in industry. Br J Ind Med 43:779–784.
6. Latko WA, Armstrong TJ, Franzblau A, et al. 1999. Cross-sectional study of the relationship between repetitive work and the prevalence of upper limb musculoskeletal disorders. Am J Ind Med 36:248–259.
7. Ljung B-O, Forsgren S, Fridén J. 1999. Substance P and calcitonin gene-related peptide expression at the extensor carpi radialis brevis muscle origin: implications for the etiology of tennis elbow. J Orthop Res 17:554–559.
8. Alfredson H, Lorentzon R. 2002. Chronic tendon pain: no signs of chemical inflammation but high concentrations of the neurotransmitter glutamate. Implications for treatment? Curr Drug Targets 3:43–54.
9. Alfredson H, Ljung B-O, Thorsen K, et al. 2000. In vivo investigation of ECRB tendons with microdialysis technique-no signs of inflammation but high amounts of glutamate in tennis elbow. Acta Orthop Scand 71:475–479.
10. Freeland AE, Tucci MA, Barbieri RA, et al. 2002. Biochemical evaluation of serum and flexor tenosynovium in carpal tunnel syndrome. Microsurgery 22:378–385.
11. Hirata H, Nagakura T, Tsujii M, et al. 2004. The relationship of VEGF and PGE2 expression to extracellular matrix remodelling of the tenosynovium in the carpal tunnel syndrome. J Pathol 204:605–612.
12. Brain SD, Cox HM. 2006. Neuropeptides and their receptors:innovative science providing novel therapeutic targets. Br J Pharmacol 147:S202–S211.
13. Ljung B, Alfredson H, Forsgren S. 2004. Neurokinin 1-receptors and sensory neuropeptides in tendon insertions at the medial and lateral epicondyles of the humerus Studies on tennis elbow and medial epicondylalgia. J Orthop Res 22:321–327.
14. Messner K, Wei Y, Andersson B, et al. 1999. Rat model of Achilles tendon disorder. A pilot study. Cells Tissues Organs 165:30–39.
15. Andersson G, Danielson P, Alfredson H, et al. 2008. Presence of substance P and the neurokinin-1 receptor in tenocytes of the human Achilles tendon. Regul Pept 150:81–87.
16. Ettema AM, Amadio PC, Zhao C, et al. 2004. A histological and immunohistochemical study of the subsynovial connective tissue in idiopathic carpal tunnel syndrome. J Bone Joint Surg Am 86-A:1458–1466.
17. Nakama LH, King KB, Abrahamsson S, et al. 2006. VEGF, VEGFR-1, and CTGF cell densities in tendon are increased with cyclical loading: An in vivo tendinopathy model. J Orthop Res 24:393–400.
18. Soslowsky LJ, Thomopoulos S, Esmail A, et al. 2002. Rotator cuff tendinosis in an animal model:role of extrinsic and overuse factors. Ann Biomed Eng 30:1057–1063.
19. Barbe MF, Barr AE, Gorzelany I, et al. 2003. Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. J Orthop Res 21:167–176.
20. Barbe MF, Elliott MB, Abdelmagid SM, et al. 2008. Serum and tissue cytokines and chemokines increase with repetitive upper extremity tasks. J Orthop Res 26:1320–1326.
21. Al-Shatti T, Barr AE, Safadi FF, et al. 2005. Increase in inflammatory cytokines in median nerves in a rat model of repetitive motion injury. J Neuroimmunol 167:13–22.
22. Clark BD, Al-Shatti TA, Barr AE, et al. 2004. Performance of a high-repetition, high-force task induces carpal tunnel syndrome in rats. J Orthop Sports Phys Ther 34:244–253.
23. Elliott MB, Barr AE, Kietrys DM, et al. 2008. Peripheral neuritis and increased spinal cord neurochemicals are induced in a model of repetitive motion injury with low force and repetition exposure. Brain Res 1218:103–113.
24. Rani S, Barbe MF, Barr AE, et al. 2009. Periostin-like-factor and Periostin in an animal model of work-related musculoskeletal disorder. Bone 44:502–512.
25. Kehl LJ, Hamamoto DT, Wacnik PW, et al. 2003. A cannabinoid agonist differentially attenuates deep tissue hyperalgesia in animal models of cancer and inflammatory muscle pain. Pain 103:175–186.
26. Wacnik PW, Kehl LJ, Trempe TM, et al. 2003. Tumor implantation in mouse humerus evokes movement-related hyperalgesia exceeding that evoked by intramuscular carrageenan. Pain 101:175–186.
27. Cook JL, Feller JA, Bonar SF, et al. 2004. Abnormal tenocyte morphology is more prevalent than collagen disruption in asymptomatic athletes' patellar tendons. J Orthop Res 22:334–338.
28. Soslowsky LJ, Thomopoulos S, Esmai A, et al. 2002. Rotator cuff tendinosis in an animal model: role of extrinsic and overuse factors. Ann Biomed Eng 30:1057–1063.
29. Rani S, Barbe MF, Barr AE, et al. 2009. Induction of Periostin-Like-Factor and Periostin in Forearm Muscle, Tendon and Nerve in an Animal Model of Work-Related Musculoskeletal

Disorder. J Histochem Cytochem. 2009. [Epub ahead of print] PMID: 19620321.
30. Ackermann PW, Li J, Lundeberg T, et al. 2003. Neuronal plasticity in relation to nociception and healing of rat achilles tendon. J Orthop Res 21:432–441.
31. Berglund M, Hart DA, Wiig M. 2007. The inflammatory response and hyaluronan synthases in the rabbit flexor tendon and tendon sheath following injury. J Hand Surg Eur 32:581–587.
32. Archambault JM, Hart DA, Herzog W. 2001. Response of rabbit Achilles tendon to chronic repetitive loading. Connect Tissue Res 42:13–23.
33. Uchio Y, Ochi M, Ryoke K, et al. 2002. Expression of neuropeptides and cytokines at the extensor carpi raidalis brevis muscle origin. J Shoulder Elbow Surg 11:570–575.
34. Rupp EA, Cameron PM, Ranawat CS, et al. 1986. Specific bioactivities of monocyte-derived interleukin 1 alpha and interleukin 1 beta are similar to each other on cultured murine thymocytes and on cultured human connective tissue cells. J Clin Invest 78:836–839.
35. Marsolais D, Cote CH, Frenette J. 2001. Neutrophils and macrophages accumulate sequentially following Achilles tendon injury. J Orthop Res 19:1203–1209.
36. Tsuzaki M, Guyton G, Garrett W, et al. 2003. IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. J Orthop Res 21:256–264.
37. Archambault J, Tsuzaki M, Herzog W, et al. 2002. Stretch and interleukin-1beta induce matrix metalloproteinases in rabbit tendon cells in vitro. J Orthop Res 20:36–39.
38. Andersson G, Danielson P, Alfredson H, et al. 2007. Nerve-related characteristics of ventral paratendinous tissue in chronic Achilles tendinosis. Knee Surg Sports Traumatol Arthrosc 15:1272–1279.
39. Reynier-Rebuffel A, Mathiau P, Callebert J, et al. 1994. Substance P, calcitonin gene-related peptide, and capsaicin release serotonin from cerebrovascular mast cells. Am J Physiol 267:1421–1429.
40. Lai J, Ho W, Zhan G, et al. 2001. Substance P antagonist (CP-96,345) inhibits HIV-1 replication in human mononuclear phagocytes. Proc Natl Acad Sci USA 98:3970–3975.
41. Weinstein J. 1991. Neurogenic and nonneurogenic pain and inflammatory mediators. Orthop Clin North Am 22:235–246.
42. Hua X, Back S, Tam E. 1996. Substance P enhances electrical field stimulation-induced mast cell degranulation in rat trachea. Am J Physiol 270:985–991.
43. Morioka N, Takeda K, Kumagai K, et al. 2002. Interleukin-1beta-induced substance P release from rat cultured primary afferent neurons driven by two phospholipase A2 enzymes: secretory type IIA and cytosolic type IV. J Neurochem 80:989–997.
44. Nilsson J, von Euler AM, Dalsgaard CJ. 1985. Stimulation of connective tissue cell growth by substance P and substance K. Nature 315:61–63.