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Exposure dependent increases in IL-1 β , Substance P, CTGF and tendinosis in flexor digitorum tendons with upper extremity repetitive strain injury

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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 $60 \pm 5\%$ maximum pulling force (MPF)), or a low repetition negligible force (LRNF) reaching and food retrieval task (3 reaches/min at $5 \pm 5\%$ MPF), for 2 hrs/day in 30 min sessions, 3 days/wk for 3–12 weeks. Forelimb grip strength was tested. Flexor digitorum tendons were examined at mid tendon at the level of the carpal tunnel for interleukin (IL)-1 β ; 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-1 β 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 declines.

Keywords

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

Introduction

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; while 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 is still under investigation. Although the presence of an inflammatory component has not been identified by all investigators (7–9), increased inflammatory molecules, e.g. PGE₂, have been found in tenosynovium of patients diagnosed with carpal tunnel syndrome (CTS), especially during the intermediate phase (10,11). However, PGE₂ 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 hyperalgesia, 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; 8 reaches/min at 5 ± 5 % MPF) for 2 hours/day, 3 days/week, animals show declines 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 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 both responses are exposure dependent (with greater tissue responses over time and with higher demand tasks). We also hypothesize that inflammatory and neurochemical tendon changes are related to declines in grip strength, a behavior test that correlates with movement-related hyperalgesia (25,26). Therefore, in this study, we examined grip strength, IL-1 β 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 in a 12 hr 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 hrs/day, in 30 min sessions, separated by 1.5 hour breaks, 3 days/wk 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 20). The task and trained rats were food-restricted and maintained within $\pm 5\%$ 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).

Determination of Grip Strength

Maximum forearm grip strength was determined bilaterally for all control and HRHF rats weekly. LRNF grip strength data was 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 are 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 3–5 times/limb, and maximum grip strength per trial included in the statistical analysis.

Measurement of tendon IL-1 β 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 hours after last task performance. The flexor digitorum tendons were separated using a scalpel from the muscle belly (in rats the flexor digitorum muscle is not clearly divided into superficial and profundus heads; therefore, we are examining the combined flexor digitorum tendons) (see Figure 3K,L), lumbrical muscles removed, then tendons rinsed in sterile saline, homogenized and lysates analyzed for IL-1 β using ELISA as described previously (20). Each sample was run in duplicate and data (pg cytokine protein) normalized to μ g 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 PO₄ buffer, and forearm musculotendinous tissues were dissected as a mass off the forearm bones as shown in Figure 3K,L, and sectioned longitudinally as a soft tissue mass (*en bloc*) as described previously (19, 23). Sections, on slides, were incubated in 3% H₂O₂ in methanol (4°C) for 30 min, washed, incubated in 4% dried milk in PBS (Blotto) for 20 min, and then overnight at rm temp 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 hrs at rm temp with goat anti-mouse peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. HRP was visualized as a black immunoreactive stain using diaminobenzidine (DAB) with cobalt (Sigma-Aldrich, St. Louis, MO). For IL-1 β and periostin like factor (PLF; labels activated fibroblasts producing this extracellular matrix 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 connective tissue growth factor (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, diluted 1:100 in PBS for 2 hrs), tissue sections were washed, digested with 0.5% pepsin in 0.01 N HCl for 20 min at rm temp, and then incubated with goat serum (4%) in PBS for 30 min at rm temp. Sections were then incubated with either anti-ED1 (MAB1435, Chemicon, Temecula, CA, 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 rm temp. Sections were incubated with appropriate secondary antibodies conjugated to Cy3 (red tag; Jackson). 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-1 β and Substance P in flexor digitorum tendons, HRP-DAB stained slides were quantified by 2 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 Figure 3K,L, for each tendon, at three microscope field locations per rat in 2–3 separate sections per rat. Para- and epitendon region measurements were summated to give peritendon measurements since 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 on 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 Figure 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 3 factors on a 4 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 are defined as: 0 (parallel fibers that are 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 20 X objective), 1 (<5 capillary profiles), 2 (6–10 capillary profiles) to 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 Figure 3K,L), for each flexor digitorum tendon at the level of the wrist, at 3 microscope field locations per rat, in 2–3 separate sections per rat.

Statistical Analyses

To test for differences in variables between the 2 control groups (normal versus 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 1 group for subsequent comparisons to experimental rats. HRHF rat grip strength and IL-1 β ELISA protein levels were analyzed by 2-way ANOVA with the factors week and limb (preferred reach and postural support). IL-1 β and CTGF immunostaining data, and neutrophil and ED-1-positive macrophage cell count data, from the preferred reach limbs was analyzed by 2-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 (3 measurements per region, per limb, and per rat) was 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 post hoc 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 ($p=0.4439$). Post hoc 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).

IL-1 β levels and neutrophils increase with HRHF task in flexor digitorum tendons

Few IL-1 β immunoreactive cells were present in control flexor digitorum tendons at the level of the wrist (Fig 3A). IL-1 β 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-1 β 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 versus endotendon). Bonferroni post hoc analyses (Fig 3E) showed that IL-1 β 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-1 β levels by week ($p=0.0022$), but not by limb ($p=0.7411$). Posthoc analysis shows that IL-1 β 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-1 β does not increase in LRNF flexor tendons compared to normal or trained controls (20).

In 6 and 12 week HRHF rats, some monocytes in peritendon blood vessels and endotendon tenocytes were IL-1 β immunoreactive (Fig 3C,D and inset in D). ED-1 stained macrophages (see Fig 5E–G for examples) 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 mid-tendon 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 clear a 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,C4). 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).

Progressive flexor digitorum tendon histopathological changes

We observed histopathological changes in HRHF 12 week flexor digitorum tendons at the level of the wrist (Fig 3B–D; Fig 4B,C; Fig 6), but not in LRNF 12 week or control flexor digitorum tendons (Fig 3A,H,I; Fig 4A; Fig 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 Fig 3B–D,J; Fig 4C), compared to 12 week LRLF and control tendons (Fig 6B,C; see also Fig 3A,H,I; Fig 4A). The increased cells in the peritendon region of HRHF rats included IL-1 β immunoreactive cells (Fig 3B–D), Substance P immunoreactive cells, axons, mast cells and macrophages (Fig 4B–D; Fig 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 wk 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 hyperplastic 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 % 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 are 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 immunostained fibroblasts and surrounding matrix increases 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-1 β immunostained cells, and IL-1 β 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 reference 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; 22). The bilateral response was due to the use of each limb in performing the HRHF task, one as the preferred reach limb and the other as their postural support limb. Since decreased forelimb grip strength is an indicator of deep tissue hyperalgesia in animal models of muscle inflammation (25,26), and the timing of IL-1 β 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 since it showed a trend for an increase at week 3 matching the onset of grip strength declines.

We observed progressive increases in the pro-inflammatory cytokine, IL-1 β , 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 with in tendons of LRNF rats (20,23). These combined findings support our hypothesis of exposure dependent tissue and behavioral responses. Increased IL-1 β has been shown to be an early response to tendon injury (21,31–33). Levels of mRNA for IL-1 β increase only transiently on day 3 in tendon and sheath after a tensile loading injury (31,32). Increased pro-inflammatory cytokines (IL-1 β and TNF α), 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 (the most likely onset of injury and associated inflammation) in these patients (10). However, in patients with lateral epicondylitis, IL-1 immunoreactive fibroblasts are observed in the extensor carpi radialis brevis muscle origin (33). IL-1 β is known to induce proliferation of fibroblasts (34). Exposing tendon cells to IL-1 β *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-1 β is most likely contributing to the initiation of the subsequent proliferative and degenerative tendon changes.

Numerous papers 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 tendinosis. Substance P has several roles including facilitating histamine release from mast cells and thus enhancing vasodilation and extravasation of immune cells (40,41). This holds true in our model as well, with both Substance P and extrinsic macrophages increasing in week 12 peritendon regions. Since IL-1 β facilitates Substance P release (42), 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 (43). 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 (17), although our increase in CTGF immunoreactive cell densities was most prominent in the peritendon versus within the tendon matrix 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 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 shows an increase in CTGF in rounded tenocytes (unpublished data). However, we have chosen to focus on flexor digitorum tendons passing through the carpal tunnel since 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. This data matches 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 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 were dependent both on level of task demands as well as on time spent engaged in an overuse activity. The timing of both tendon IL-1 β and Substance P increases suggests that they contribute to grip strength declines. Finally, the early increase of tendon IL-1 β , 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.

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Figure 1.

Cartoon of 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 msec. *D)* Rat retrieves foot pellet reward by mouth from food trough.

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.

Figure 3.

Inflammatory response in longitudinal sections of flexor digitorum tendons at wrist level. (A–D) IL-1 β immunoreactive cells in tendons of controls (A), 3 week HRHF (B), 6 week HRHF (inset shows peritendon) (C), and 12 week HRHF (D; inset shows higher power of peritendon blood vessel indicated by *). Arrows indicate IL-1 β stained fibroblasts (tenocytes). Eosin counterstain. (E) Quantification of IL-1 β 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 week HRHF rats. Mean and SEM data shown. (F) Elisa results of IL-1 β levels in preferred reach and postural support flexor digitorum tendons of normal control (C) and week 6 and 12 week HRHF rats. Mean and SEM data 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 *. (K,L) 1X macroscopic 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 μ m. bv = blood vessel; Endo = endotendon; Peri = peritendon; Para = paratendon region.

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-headed 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 shown. *:p< 0.002 compared to controls. Scale bars = 50μm. Endo = endotendon.

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 * are in insets. (C,D) Substance P immunostaining (black) in 12 week HRHF tendons counterstained with eosin showing axon like 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 μ m.

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 shown. (B–F) H&E stained tendons showing control (B and higher power inset of area indicated by *), 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 μ m.

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 *. (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 week HRHF rats. Mean and SEM data 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 asterik indicates region enlarged in inset, which shows PLF immunostaining in matrix surrounding PLF-stained fibroblasts. Endo = endotendon; Epi = epitendon; Peri = peritendon. Scale bars = 50µm.