Cyclical articular joint loading leads to cartilage thinning and osteopontin production in a novel in vivo rabbit model of repetitive finger flexion

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Summary

Objective: An in vivo rabbit model of repetitive joint flexion and loading was used to characterize the morphological effects of cyclical loading on articular cartilage.

Design: The forepaw digits of eight anesthetized New Zealand White adult female rabbits were repetitively flexed at 1 Hz with a mean peak digit load of 0.42 N for 2 h per day for 60 cumulative hours. Metacarpophalangeal joints were collected from loaded and contra-lateral control limbs, fixed, decalcified, embedded, and thin-sectioned. Serial sections were stained for histology or for immunohistochemistry. Morphometric data including the mean thicknesses of the uncalcified cartilage and of the calcified cartilage were collected from digital photomicrographs of safranin O-stained sections. The number of cells stained with osteopontin (OPN) antibody was counted.

Results: We observed a decrease in uncalcified cartilage mean thickness with no significant change in calcified cartilage thickness. We also observed a significant increase in the number of cells positive for osteopontin (OPN) in the uncalcified cartilage. These changes occurred without overt cartilage surface degeneration.

Conclusions: Cyclical loading leads to changes at the tissue and cellular levels in articular cartilage. These changes are suggestive of tidemark advancement and may indicate a reactivation of cartilage mineralization steps analogous to endochondral ossification. This novel in vivo rabbit model of repetitive flexion and loading can be used to investigate the effects of cyclical loading on articular joints.

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Key words: Animal model, Mechanical loading, Cartilage biology, Remodeling, Osteopontin, In vivo.

Introduction

Multiple risk factors for osteoarthritis (OA) have been identified including familial OA, advanced age, obesity, prior injury, and occupation (reviewed by Felson). The development and the progression of OA are mediated by multiple factors including genetics, epigenetics, biochemistry, and mechanics. Understanding the specific role of each factor is important for treating OA and is vital for creating effective therapeutic interventions. However, measuring the individual contribution of each of these factors in human subjects is complicated by the slow development of disease, the variability in symptom manifestation, and the difficulty of obtaining complete subject histories. In part to avoid these complications, in vitro and in vivo studies have been implemented to measure some of the specific factors that affect OA. Mechanical factors such as compressive, tensile, and shear loading play important roles in articular joint health and disease. These have been studied using in vitro loading experiments (reviewed by DiMicco et al.). Mechanical loading has also been studied using in vivo animal models. In some animal models joint loading is altered by surgically modifying the joint (e.g., ligament transection, full or partial meniscectomy, osteotomy, or implantation of rods) often to create a slowly progressive degeneration similar to OA. In non-invasive models, the effects of cyclical loading are evaluated by joint immobilization or by training the animal to walk or run, however, such methods cannot precisely control animal compliance or cycle frequency. These issues are overcome in the repetitive impulsive loading rabbit model (RIL model). However, the manner of loading simulates a sudden impact rather than the normal motions of joint flexion.

At this time, the beneficial and/or deleterious effects of non-traumatic compressive, tensile, and shear loading in combination are unclear. Such effects are relevant to the understanding, intervention, and prevention of degenerative joint diseases such as OA. For example, determining the relationship between compressive load levels and joint injury or between cumulative compression and injury may lead to recommendations for sports or workplace activities that can prevent injury.
Our hypothesis is that physiologic, in vivo cyclical loading causes structural and metabolic changes in cartilage. To test this hypothesis, we created a novel in vivo rabbit model of precise repetitive joint flexion and loading and measured morphometric features of the articular cartilage and the presence of osteopontin (OPN) in both the loaded and non-loaded, contra-lateral control joints.

Method

STUDY DESIGN OVERVIEW

We developed a rabbit model in which the metacarpophalangeal (MCP) joint is repetitively flexed and loaded through electrical stimulation of the large finger flexor. The rabbit was selected because a rabbit’s paw is similar to the human hand in general anatomy and tissue structures (e.g., bones of the digit, tendon attachments) when scaled for size. Rabbits have been used as in vivo models for human hand musculoskeletal disorders and for testing hand surgery techniques. Eight female, adult New Zealand White rabbits (3.5 – 4.0 kg) were loaded in individual sessions. Estimated age of the rabbits was approximately 6 months at the start of the study. The loaded in vivo digit flexion force of 0.42 N was equivalent to 17.5% \( P_0 \), based on a pilot tetanic muscle force (\( P_0 \)) experiment (\( n = 4 \) rabbits, between rabbit standard deviation (SD) ± 0.05 N). This test was analogous to the maximum voluntary contraction (MVC) commonly used to measure muscle strength in humans.

Biomechanical modeling of finger joint force by Chao et al. predicted larger shear forces for the MCP compared to the other two joints and similar compressive forces for the MCP and proximal interphalangeal (PIP). Because the MCP joints were slightly larger than the PIP, the compressive stress was lower in the MCP than in the PIP. However, the larger size of the MCP provided more experimental tissue than the other joints. Therefore, the MCP joints were examined in this study.

HISTOLOGY

Rabbits were euthanized and the MCP joints of the third digit were harvested from both limbs after 60 cumulative hours of loading were completed. These specimens were fixed in formalin, decalcified in ethylenediaminetetraacetic acid 2 or 3 weeks, dehydrated, embedded in paraffin, sectioned in the sagittal plane (\( 7 \) \( \mu \)m), and mounted on microscope slides with three sections per slide. Thin sections of every fifth slide were stained with safranin O/fast green/iron hematoxylin. Chemicals and supplies were purchased from Fisher Scientific International, Inc. (Hampton, NH), Electron Microscopy Sciences (Hatfield, PA), or Sigma-Aldrich, Co. (St. Louis, MO) unless otherwise indicated.

IMAGE ANALYSIS

Specimen handling, slide preparation, digital microphotography and image analysis were performed blinded to limb loading status.

To quantify morphological features, six of the stained slides from the central region of each MCP specimen were selected. Microscopic digital images of stained sections were captured for image analysis (Axioskop 2 MOT microscope and AxioCam digital camera, Carl Zeiss, Germany). A region of interest (ROI, 300 \( \mu \)m \( \times \) 500 \( \mu \)m) was outlined on each image on the palmar side of the distal end of the metacarpal bone within \( \sim 500 \mu \)m of the joint border. All procedures were in compliance with the principles of “Guide for the Care and Use of Animals,” and received prior approval and oversight from the University of California’s Care and Use of Animals Committee.

IN VIVO RABBIT JOINT LOADING MODEL

The rabbit was anesthetized with isofluorane (~3% continuous, Henry Schein, Inc., Melville, NY) by mask (Anesthesia Service and Equipment, Inc., Jacksonville, FL) and placed supine on a heated pad with forelimbs supported at the mid-dorsal surface leaving the digits free. Vital signs were monitored continuously (EEG pulse oximeter, Heska Corp., Fort Collins, CO). A repetitive gripping motion was induced in one paw by electrical stimulation of the FDP muscle via a 33g stimulation needle placed subcutaneously in the mid-forelimb halfway between the wrist and elbow. A Grass-Telefactor stimulator (West Warwick, RI) was used to control muscle stimulation (1 train/s of 200 ms duration, 100 pulses/s of 1.0 ms duration). Only the FDP muscle was stimulated. A sham stimulation needle was inserted into the contra-lateral control limb but was not connected to the stimulator. Load was added to the tip of the third digit by sliding a lightweight brass cuff over the digit tip and connecting the cuff to a load cell with a stiff wire. The load cell output was displayed continuously at 1000 Hz on a computer monitor by a custom Labview program (National Instruments Corp., Austin, TX). The output was saved to computer disc periodically. The stimulation voltage was adjusted manually to maintain the target mean peak digit force. Following the experiments, the force histories from 3 randomly sampled days for each rabbit were averaged, and the actual mean peak force at the digit tip was determined to be 0.42 N.

The digit tip flexion force of 0.42 N was equivalent to 17.5% \( P_0 \), the load was adjusted manually to maintain the target mean peak digit force. Following the experiments, the force histories from 3 randomly sampled days for each rabbit were averaged, and the actual mean peak force at the digit tip was determined to be 0.42 N.

The digit tip flexion force of 0.42 N was equivalent to 17.5% \( P_0 \), based on a pilot tetanic muscle force (\( P_0 \)) experiment (\( n = 4 \) rabbits, between rabbit standard deviation (SD) ± 0.05 N). This test was analogous to the maximum voluntary contraction (MVC) commonly used to measure muscle strength in humans.
thicknesstransformed into the respective area divided by the width of the ROI (500 µm).

IMMUNOHISTOLOGY

Thin sections were pretreated with hyaluronidase (Type IS, 600 U/ml in phosphate-buffered saline (PBS), pH 5.0, 10 min, 37°C) to improve antigen accessibility, followed by treatment with hydrogen peroxide to quench endogenous peroxidase activity (1% v/v in PBS, pH 7.2, 10 min, room temperature). Non-specific binding sites were blocked in diluted normal serum (30 min, room temperature) prior to overnight incubation with primary anti-OPN antibody (1 µg/ml at 4°C). Antigen–antibody binding was identified following manufacturer’s protocols using horseradish peroxidase and Nova Red substrate (VectaStain ABC, Vector Lab, Burlingame, CA). The protocol for the negative control immunochemical experiment was identical but that the primary antibody was omitted. The sections from one rabbit were damaged and not analyzed.

The monoclonal anti-OPN antibody, “2A1,” was a kind gift of Dr. David Denhardt, Rutgers University. The antibody was prepared using purified recombinant OPN as described by D’Alonzo et al.18 when used on histological preparations the antibody recognizes a highly conserved epitope in the C-terminal half of the protein.19,20

Digital microphotographs were captured as described above. The number of cells with antigenic staining and the number of cells without antigenic staining were counted. Cell density was calculated.

QUALITATIVE ANALYSIS

Other histological details related to joint pathology were noted for the cartilage of the entire joint. These included the presence of cartilage surface delamination or fibrillation, osteophyte formation, tidemark duplication, blood vessels in the cartilage, and safranin O staining density.

DATA ANALYSIS

The morphometric data were summarized as the means of six sections per joint for each outcome measure. These means appeared normally distributed. The means are reported ± SD of the mean. The OPN data were from one section per joint. For the determination of statistical significance, two-tailed, paired Student’s t-tests were used to compare loaded and contralateral control specimens (n = 8 rabbits for morphometric measures, n = 7 rabbits for OPN). The statistical significance level (α) was ≤ 0.05. Tests were performed using Sigma Plot v. 8.1 (Systat Software, Inc., Richmond, CA).

Results

GROSS HISTOLOGICAL FINDINGS

Typical histologic findings from the experimental and control joints of one rabbit are presented in Fig. 1. The articular cartilage surfaces of all sections were intact without delamination or fibrillation including the regions not used for quantitative findings. The cartilage tissue of all sections stained evenly with safranin O, suggesting that a net loss of proteoglycans did not occur. Osteophytes were not observed. A duplicated tidemark was noted in at least one section of most specimens (seven of eight joints from both control and loaded limbs). A blood vessel either crossing or abutting the osteochondral junction was noted in at least one section of most specimens (seven of eight joints from control and loaded limbs). No blood vessels were observed in the uncalcified cartilage of either limb.

MEAN THICKNESS OF UNCALCIFIED AND CALCIFIED CARTILAGE

The uncalcified cartilage mean thickness and the calcified cartilage mean thickness were measured individually (Table I). The change in calcified cartilage mean thickness did not meet our statistical criteria (α ≤ 0.05). Total cartilage mean thickness (calculated as the sum of the uncalcified and the calcified mean thicknesses; P = 0.08) also did not meet our statistical criteria since these data were diluted by the calcified data. However, the decrease in the uncalcified cartilage mean thickness (P = 0.03) did meet our statistical criteria. In seven of eight rabbits the uncalcified cartilage mean thickness was less in the loaded limb compared to the contra-lateral control limb (Fig. 2).

OSTEOPONTIN

In the uncalcified cartilage, OPN staining was localized to the cell and its pericellular matrix (Fig. 3). In half of the joints some staining occurred at the joint surface, but this did not appear to be related to load status. No cells stained when the 2A1 antibody was omitted in the negative control immunohistochemistry experiments.

The number of cells staining positive for OPN was greater in the loaded limb of seven rabbits compared to the control limb. The number of cells with antigenic staining and the number of those without were counted, and the density of OPN-stained cells was calculated. The mean number of positive cells per mm² of uncalcified cartilage was 0.89 (±0.7) in the control joint and 1.50 (±0.9) in the loaded joint (P = 0.01). The subject number was reduced because the control sections for rabbit 4 were damaged and not suitable for quantitative analysis.

We noted a difference in the baseline intensity of staining between rabbits. In the unloaded, control sections, overall staining was less intense for rabbits 1, 2, 3, and 4 than for rabbits 5, 6, 7, and 8. In the control sections, the mean density of positive cells for rabbits 1–3 was 0.25/mm² (±0.27), while for rabbits 5–8 the mean density positive was 1.37 cells/mm² (±0.41). The only difference in the treatment of specimens was that joints from rabbits 1–4 were decalcified for 2 weeks, while joints from rabbits 5–8 were decalcified for 3 weeks. The additional week of decalcification may have enhanced immunocytochemical staining with this antibody. However, since paired control and loaded joints from individual rabbits were decalcified and processed at the same duration, the difference in duration of decalcification did not affect the percent increase in the number of positive cells with loading. The increase with loading in the joints of rabbits 1–3 (68.4% ± 39.3) was similar to that of rabbits 5–8 (mean 70.1 ± 51.4). The percent increase in the density of OPN-positive cells for all seven rabbits was significant (mean 69.4% ± 42.9, P = 0.01).

Discussion

This is the first in vivo study to control the levels of both load and frequency of cyclical loading of articulating joints.
Sixty cumulative hours of physiological loading leads to both structural (uncalcified cartilage thinning) and metabolic (increased OPN staining) changes to the cartilage.

There are three plausible mechanisms for the observed cartilage thinning. The first is tissue loss via degradation. However, we observe no cartilage surface delamination, no fibrillation, and no qualitative loss of proteoglycan staining (safranin O) in loaded joints. The histological methods used do not measure synthesis of proteoglycan which could maintain safranin O staining levels despite minor degradation. Increased synthesis of proteoglycan occurs with dynamic loading in vitro and could be an effect of loading in the present study as well.

A second explanation is condensation of the tissue. This has been observed by others using in vitro cyclical compressive loading of osteochondral explants. The change in cartilage volume may have been due to fluid loss from the unconfined explants. A quantitative in vivo magnetic resonance imaging (MRI) study demonstrates that dynamic knee bending decreases human patellar cartilage volume. This volume decrease reverses after 90 min of rest, which the authors attribute to temporary fluid loss and return. Temporary fluid loss as a cause of tissue condensation in our model is unlikely since loading occurred over 10 weeks. Studies are in progress to determine if compaction of the collagen network is

Table I

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<thead>
<tr>
<th>Morphologic measures of articular cartilage after cyclical loading (n = 8 rabbits)</th>
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<tr>
<td>Control limb ± SD</td>
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<tr>
<td>Uncalcified cartilage mean thickness (µm)</td>
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<tr>
<td>Calcified cartilage mean thickness (µm)</td>
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<tr>
<td>Total articular cartilage mean thickness (µm)</td>
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<td>Uncalcified cartilage portion of total mean thickness (%)</td>
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*Percent change values are the means of the differences between experimental and control.
†Significance was determined using two-tailed paired t tests between control and experimental limbs.
a contributing factor in uncalcified cartilage thinning in our model.

A third possible mechanism for uncalcified cartilage thinning is progressive mineralization of the uncalcified cartilage, sometimes called “tidemark advancement.” The tidemark is a division in cartilage between the uncalcified and the calcified regions that continues to advance toward the joint surface, but very slowly, throughout life. It has been demonstrated that mechanical load does affect mineralization. Lane and Bullough demonstrate that tidemark advancement and subchondral plate remodeling via increased vascularity occur to a greater extent in regions of human humeral and femoral heads expected to be more highly loaded. We have not observed a net increase in vascularity with our methods. The shaved surface method used by Lane et al. may be more sensitive to vascular measurement, but this method would inhibit our ability to perform immunohistology on serial sections.

If the tidemark moves from the calcified cartilage into the uncalcified, one might expect the mean thickness of the calcified cartilage to increase. For example, impact loading has been found to thicken calcified cartilage at the expense of the uncalcified cartilage. In the present study, the
Intermittent joint loading and motion causes fluid flow to examine the effect of mechanical load on OPN in adult Gly-Asp30. In the present study, the total number of OPN-collagenous, sialic acid-rich phosphorylated matrix protein loading causes an increase in cellular OPN, a non-significant \( (P < 0.05) \) decreasing, but our observation was not statistically significant \( (P = 0.37) \). This lack of significance may be attributed to measurement error introduced by the undulation of the osteochondral interface. It should also be noted that the osteochondral interface itself may be remodeling with load.

With the present data, we are unable to state if one interface is moving at a faster rate than the other. If the tidemark were moving faster than the osteochondral interface, then the proportion of cartilage that is uncalcified would decrease significantly while that proportion which is calcified would increase. In the present study, such effects on proportions are not significant \( (P = 0.32) \). Therefore, the interface movement rates are either identical or similar enough to be indistinguishable with the present methods.

At present, tidemark advancement is one of three potential explanations that are not necessarily mutually exclusive. However, evidence to support a revival of endochondral ossification such as identification of mineralization markers is required.

Mineralization of the uncalcified cartilage would likely involve a change in phenotype of at least some of the uncalcified cartilage chondrocytes29. In our model, cyclical loading causes an increase in cellular OPN, a non-collaggenous, sialic acid-rich phosphorylated matrix protein that contains the cell-binding amino acid sequence, Arg-Gly-Asp30. In the present study, the total number of OPN-positive cells as well as the number of positive cells per mm² increases with loading. We found a consistent increase in the percentage of cells with OPN staining in both groups, implying metabolic changes in the cells are occurring in response to mechanical loading. However, because the exact number of OPN-positive cells in the unloaded control joint can only be estimated, more investigation into the effect of decalcification should be performed. This response to mechanical loading has been demonstrated in vivo in bone31,32. Studies using bone cells in culture show increased OPN expression and synthesis due to loading, specifically to hydrostatic compressive loading33 and to fluid flow34,35. Mitogen-activated protein kinases (MAPK) likely transduce the fluid flow stimulus for OPN gene expression in bone cells36. Our study is the first to examine the effect of mechanical load on OPN in adult articular chondrocytes. Here, too, fluid flow is relevant. Intermittent joint loading and motion causes fluid flow particularly in the upper cartilage zones36. We speculate that a signaling mechanism similar to that of bone may be involved. However, the exact role of OPN in cartilage is unknown.

The expression of OPN is thought to be inhibited in non-hypertrophic chondrocytes due to a negative promoter element as demonstrated in transgenic mice37. However, chondrocyte expression of OPN messenger RNA (mRNA) does occur in distraction osteogenesis in which tensile strain (0.5 mm/day) is applied to the healing cartilaginous callus resulting in endochondral ossification and subsequent bone formation38. Furthermore, OPN is produced by the chondrocytes of human OA cartilage with a correlation between OPN mRNA and the presence of cellular and territorial matrix OPN protein39. To answer whether the repetitive flexion and load-induced tidemark advance and OPN production are simply physiological adaptations or ultimately lead to pathological changes will require experiments of longer duration.

A disadvantage of our animal model is that under general anesthesia the skeletal muscles are flaccid40, so there is no co-contraction and there is no muscle activity during digit extension (gravity and passive joint forces return the digits to resting posture). Due to the anesthesia, the loads are very similar, but not identical, to conscious repetitive motor activities. However, this model does allow precise control of mean peak load and repetition frequency, as well as control of other parameters such as rate of load, duty cycle, and total duration. This new in vivo model will not only aid our fundamental understanding of the mechanobiology of articular joint loading, but has the potential to advance our understanding of adult ossification by testing related hypotheses. For example, Carter et al.41,42 postulate that shear stress promotes, but hydrostatic stress inhibits, mineralization. It may be that in our in vivo cyclical loading model a disruption of the normal ratio between intermittent shear stresses and intermittent hydrostatic stresses is responsible for accelerated tidemark advancement and subsequent thinning of the uncalcified cartilage.

Future studies using this model will benefit the interpretation of environmental impact on human OA studies, particularly the impact of subjects’ occupational and athletic activities. This model also has the potential for exploring more damaging parameters such as higher force, greater frequency, and longer duration of joint loading. This may allow us to determine thresholds for injury (i.e., good loading vs bad loading) as suggested by in vitro studies43. Finally, because any successfully engineered tissue for joint repair must be functional for many years, knowledge of the in vivo effects of loading is critical. Future studies using this model will potentially benefit the design of joint tissue constructs and the design of post-implant therapy.

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References