Alteration in articular cartilage of rat knee joints after spinal cord injury

† School of Health and Social Services, Saitama Prefectural University, Koshigaya-shi, Saitama 343-8540, Japan
‡ Hiroshima City Counseling Center for the Physically Disabled, Hiroshima-shi, Hiroshima 732-0052, Japan
§ Graduate School of Health Science, Hiroshima University, Hiroshima-shi, Hiroshima 734-8551, Japan
‖ Department of Physical Therapy, Ryotokuji University, Urayasu-shi, Chiba 279-0014, Japan

Summary

Objective: Mechanical forces are crucial for the maintenance of the morphologic and functional integrity of articular cartilage. The alteration of the articular cartilage after spinal cord injury (SCI) has been described in relation to a suppression of mechanical forces, since the joint is unloaded and restricted in movement. However, the morphological and biochemical characteristics of the cartilage after SCI are still poorly understood. We identified the localization of cartilage alterations after SCI and verified the influence of mechanical forces on the articular cartilage.

Method: A total of 32 Wistar rats were used. Sixteen animals underwent an SCI and 16 animals served as control. The articular cartilage of the knee joint was assessed, respectively, at 4, 8, 10, and 12 weeks after intervention by histochemical, histomorphometric, immunohistochemical, and biochemical analyses.

Results: Cartilage thickness of spinal cord-injured knees decreased at the tibial and posterior femoral (FP) regions and increased at the anterior femoral (FA) region. Spinal cord injuries decreased the number of chondrocytes at the anterior regions and decreased the cartilage matrix staining only at the tibial regions. Immunolabeling to collagen type II was noted comparably in the superficial layer but noted weakly from the middle to deep layer. Collagen type I existed excessively at the cartilage surface and the pericellular regions.

Conclusion: Cartilage alterations after SCI would not be explained by only a suppression of mechanical forces by unloading and immobilization, but there may be influences on the cartilage in addition to the change in mechanical forces.

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Key words: Spinal cord injuries, Cartilage, Paralysis, Rats, Knee joint.

Introduction

Mechanical forces such as loading and movement are a prerequisite for the development, aging, and maintenance of the morphologic and functional integrity of articular cartilage. Many animal studies have documented that unloading and immobilization cause multiple alterations to articular cartilage including increased or decreased or unchanged thickness of cartilage, increase or decrease in the number of chondrocytes, reduced proteoglycan content, and elevated or unchanged collagen content. The results of these animal studies have indicated that cartilage alterations differ between cartilage regions, based on mechanical forces specific for each region. In addition, O’Connor confirmed different patterns of alterations between with and without immobilization during unloading.

In patients with spinal cord injury (SCI), the lower limb joints are unloaded and restricted in movement. Therefore, cartilage alterations after SCI have been described in relation to alterations obtained on various animal models with the suppression of mechanical forces by unloading and immobilization. There have been suggestions that the articular structures of these patients exhibit contractions, osteoarthritides, alterations of the periarticular connective tissue, joint space narrowing, overgrowth of the epiphyses, periaricular osteoporosis, joint effusion, and heterotopic ossification. Only three prior studies have directly assessed the influence of SCI on the articular cartilage. Enneking and Horowitz observed histologically on the sagittal section stained with hematoxylin and eosin in a human knee joint with SCI of 3 and 1-1/2 years’ duration and found that the articular cartilage was normal. Unlike this report, Vanwanseele et al. identified the progressive thinning of the human knee cartilage after SCI. These annual changes exceeded the changes during normal aging by a factor of 20:1 and the changes reported in patients with osteoarthritis by a factor of 2:1. Owing to the discrepancies among these results so far, alterations of the articular cartilage after SCI are controversial and remain elusive.
The primary goal of this study was to identify the localization of cartilage alterations after SCI. To reach our goal, we examined the knee cartilage of a rat model with SCI that mimics the natural and longitudinal process in humans with SCI in the absence of normal joint loading and movement, and, applied histochemical, histomorphometric, immunohistochemical, and biochemical techniques to verify the influence of mechanical forces on the articular cartilage. To quantify histologic alterations of cartilage regions which were determined by mechanical forces associated with joint loading and movement, we selected the following three histologic characteristics: thickness of the articular cartilage, number of chondrocytes, and matrix staining to toluidine blue as a reflection of proteoglycan content. Furthermore, we assessed the distribution and content of collagen which is key to the understanding of cartilage alterations using immunoassay analysis.

Materials and methods

EXPERIMENTAL DESIGN

This study was carried out in accordance with the guide for the institutional committee of laboratory animals. Thirty-two 8-week-old female Wistar rats (CLEA Japan Inc., Tokyo, Japan), weighing 162–194 g, were chosen. Four animals in the SCI group and two each in the sham- and nonoperated groups formed a one-time cohort. Four-time cohorts were assessed relative to the time after intervention, and they were 4, 8, 10, and 12 weeks. The right and the left knee joints served as different samples. The animals were maintained under artificial conditions at 23 ± 1°C, with a constant humidity of 55 ± 5%, a cycle of 12 h light and 12 h dark, and free access to food and tap water.

SURGICAL PROCEDURES AND POSTOPERATIVE CARE

Surgical procedures and postoperative care conformed to our previous reports. The 16 SCI animals were anesthetized by intraperitoneal administration of 40 mg/kg sodium pentobarbital. After the spinal cord was exposed by a laminectomy of the T8 vertebra, it was completely transected. While under general anesthesia, the animals were perfused from the ascending aorta with 4% paraformaldehyde in phosphate buffered saline at pH 7.4. The knee joints including the patella and joint capsule were resected and the fixed specimens were immersed in 4% paraformaldehyde at 4°C. After decalcification, the specimens were embedded in paraffin and 8-μm-thick sections were cut. The fixed specimens were then stained with hematoxylin and eosin (H&E), safranin-O and fast green, andovsky blue and azure II. The examinations were performed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) under phase-contrast microscopy. The thickness of the cartilage was calculated by dividing the area of the cartilage by the number of cells within a given area. The number of chondrocytes was calculated by the total number of chondrocytes per square millimeter. Chondrocyte cisternae with pyknotic or absent nuclei were interpreted as chondrocyte death (by apoptosis or necrosis) and were not counted. Decreased matrix density with toluidine blue staining strongly correlates with a decrease in proteoglycan content. Under standardized light conditions, immunohistochemical sections stained with toluidine blue were digitized as per the measurement of cartilage thickness. At each of the four regions, the density of cartilage matrix staining with toluidine blue was measured with the Image Tool software (Scion Image Beta 4.03; Scion Corporation, Frederick, MD, USA). The mean thickness of the cartilage was calculated by dividing the area by its width, that is, by 1 mm. When the area contained noncartilaginous tissues, the noncartilaginous mean thickness was measured separately.

Histologic sections stained with safranin-O fast green were digitized by a 20× microscope objective with a light microscope and a camera. The standardized rectangular field (90 μm deep and 400 μm long) was superimposed over the histologic sections. At each of the four cartilage regions, we counted manually chondrocytes within the rectangular field. The cell density was calculated by the total number of chondrocytes per square millimeter. Chondrocyte lacunae with pyknotic or absent nuclei were interpreted as chondrocyte death (by apoptosis or necrosis) and were not counted. Decreased matrix density with toluidine blue staining strongly correlates with a decrease in proteoglycan content. Under standardized light conditions, immunohistochemical sections stained with toluidine blue were digitized as per the measurement of cartilage thickness. At each of the four regions, the density of cartilage matrix staining with toluidine blue was measured with the Image Tool software. Intense metachromatic areas at 30 evenly spread points were counted. The average staining intensity of the 30 points constituted the matrix staining.

COLLAGEN IMMUNOHISTOCHEMICAL ANALYSIS

We assessed the distribution of collagen type II and type I in the articular cartilage using enzyme-conjugated polymer techniques. When the medial mid-condylar histologic sections were used, the whole surface of femoral, tibial, and patellar cartilage failed to be identified simultaneously over the same section. Therefore, on standardized lateral mid-condylar sections, we measured the distribution and content of collagen which is key to the understanding of cartilage alterations using immunoassay analysis.
observed the whole surface of these cartilages. Deparaffinized sections were treated with 0.5% bovine testicular hyaluronidase (Sigma–Aldrich Co., in tris buffered saline (TBS) (pH 7.6) for 60 min at 20°C. They were incubated for 18 h at room temperature with either rabbit polyclonal anti-rat collagen type II (1:500) or type I (1:1000) antibody (NovoTec, Lyon, France) diluted in TBS with 3% bovine serum albumin (BSA) and 1% normal goat serum. Nonspecific binding was eliminated with 3% BSA and 0.9% NaCl in TBS for 20 min at room temperature, and endogenous peroxidase was inactivated with 3% BSA and 5% H2O2 in TBS for 20 min. The slides were then incubated with ENVISION +–, Rabbit/HRP (Dako Japan Inc., Tokyo, Japan) for 45 min at room temperature. Color was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB +–; Dako Japan Inc.). Sections were finally counterstained with Harris’s hematoxylin, washed in water, and coverslipped.

Control staining was performed by omitting the primary antibody. All slides were stained for each primary antibody in one session (same day, same reagents, and same protocol). The slides were randomly labeled, and the observer was blinded as to which cartilage was examined.

### RESULTS

#### ANIMALS

Rats with SCI crawled and dragged the hindlimbs throughout the experimental period. The animal moved around the cage by walking on its forelimbs and unable to support weight on plantar placement. The knee after SCI was in a more flexed position, as disclosed by our previous reports, and the hindlimbs’ dragging loaded the anterior knee. Kick movements, which were defined as spasticity, were often observed after 2 weeks. Involuntary slight movements of hip and/or knee joints were apparent after 4 weeks. After that, we found no further changes in the hindlimb movement until 12 weeks.

All animals demonstrated normal behavior before surgery, and the sham-operated animals had no observable deficits at any time during the experimental period after laminectomy as well as the nonoperated animals. For all outcome measures, given the lack of differences between sham- and nonoperated knees (confidence interval including the null), the results were combined to form one comparison (control) group against which the effects of SCI were examined.

#### CARTILAGE HISTOMORPHOMETRY

There were no fissures, erosions, or abrasions in the femoral, tibial, and patellar articular cartilage. The mean cartilage thickness for the FA, FP, TA, and TP regions are shown in Table I. Articular cartilage at the FA region was significantly thicker in the SCI group than in the control group at all time points [4 weeks, 26%, power = 94.6; 8 weeks, 65%, power = 99.97; 10 weeks, 57%, power = 99.6; 12 weeks, 62%, power = 99.9; P < 0.05; Fig. 2(A,B)]. Conversely, the FP cartilage was thinner in SCI rats than in control rats at all time points [4 weeks, 32%, power = 99.9; 8 weeks, 31%, power = 99.4; 10 weeks, 35%, power = 99.4; 12 weeks, 33%, power = 99.97; P < 0.05; Fig. 2(C,D)], and the TA and TP cartilage was thinner in SCI rats at 8 and 12 weeks (8 weeks, 18% and 22%, both power = 96.0; 12 weeks, 20%, power = 86.5 and 29%, power = 99.9; respectively, P < 0.05). Although the cartilage thickness showed no significant difference among any time points at the FP, TA, and TP regions in the SCI group (P > 0.05), time had a statistically significant effect on the cartilage thickness at the FA region (P = 0.02). The FA thickness significantly increased between 4 and 8 weeks and decreased between 8 and 12 weeks. On microscopic examination, the subchondral bone penetrating into the cartilage was observed at the FP, TA, and TP regions, especially at the FP region. While the

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**Table I**

<table>
<thead>
<tr>
<th>Regions</th>
<th>Groups</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>10 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>Control</td>
<td>141.5 (131.9, 151.5)</td>
<td>128.6 (120.9, 154.8)</td>
<td>150.8 (142.9, 158.9)</td>
<td>125.7 (105.8, 163.4)</td>
</tr>
<tr>
<td>SCI</td>
<td>178.1 (171.4, 185.3)</td>
<td>221.6 (201.1, 232.9)</td>
<td>204.5 (183.6, 225.2)</td>
<td>256.8 (250.1, 325.2)</td>
<td>267.8 (241.0, 306.5)</td>
</tr>
<tr>
<td>FP</td>
<td>96.0 (183.6, 215.5)</td>
<td>182.0 (168.4, 224.4)</td>
<td>177.2 (163.3, 212.5)</td>
<td>202.7 (173.6, 216.9)</td>
<td>202.7 (173.6, 216.9)</td>
</tr>
<tr>
<td>TA</td>
<td>273.8 (243.1, 281.4)</td>
<td>269.7 (260.4, 274.3)</td>
<td>261.7 (249.3, 342.7)</td>
<td>325.1 (280.1, 343.2)</td>
<td>325.1 (280.1, 343.2)</td>
</tr>
<tr>
<td>TP</td>
<td>238.7 (213.3, 270.1)</td>
<td>220.5 (192.4, 244.9)</td>
<td>264.4 (177.6, 310.2)</td>
<td>262.7 (212.0, 277.5)</td>
<td>262.7 (212.0, 277.5)</td>
</tr>
</tbody>
</table>

Displacement values are given as median (range) μm.

*Cartilage was significantly thicker in the SCI group than in the control group.

**D** CARTILAGE BIOCHEMICAL ANALYSIS

We assessed the levels of collagen type II and type IX in the rat articular cartilage using Western blot. At the end of the maintenance intervals, the animals of half the number in each time cohort were euthanized with an overdose of diethyl ether. Cartilage samples of the femur, tibia, and patella were pulverized in liquid nitrogen and homogenized in 5 × volume/wet-weight of ice-cold homogenization buffer containing 0.05 M Tris–HCl (pH 7.4), 0.15 M NaCl, 1% Triton X-100, and protease inhibitors (Complete, protease inhibitor cocktails; Roche Diagnostics, Basel, Switzerland). The bicinechonic acid protein assay was used to determine the total protein concentration in the cartilage samples. Protein samples (10 μg each) were separated electrophoretically on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Slab Electrophoresis Chamber AE-6200; ATTO Co., Tokyo, Japan) and transferred onto a polyvinylidene difluoride membrane with a semi-dry blotting apparatus (HorizBlot AE-6677; ATTO Co.) The blots were incubated with either the goat polyclonal anti-human collagen type I (I;I:500; Santa Cruz Bio-technology, Inc., Santa Cruz, CA, USA) or the mouse monoclonal anti-human collagen type IX (I;I:2500; Daiichi Fine Chemical Co., Toyama, Japan) in TBS-Tween for 60 min at room temperature. Horseradish peroxidase-labeled donkey anti-goat IgG for collagen type II (I;I:25,000; Santa Cruz Biotechnology, Inc.) or sheep anti-mouse IgG for collagen type IX (I;I:5000; Amersham Biosciences, Buckinghamshire, England) was used for 60 min at room temperature. The reaction was developed with the enhanced chemiluminescence assay (ECL–Western Blotting Detection System; Amersham Biosciences). The signals were analyzed by measuring the densities of the immunoreactive bands relative to the sample in the nonoperated group of 4 weeks with the Image J software.

### STATISTICAL ANALYSIS

Statistical analyses were conducted with SPSS 11.5J for Windows (SPSS Japan Inc., Tokyo, Japan). All values in the text and table are presented here as median and range. Results of histomorphometry and Western blot were given the lack of differences between sham- and nonoperated knees (confidence interval including the null), the results were combined to form one comparison (control) group against which the effects of SCI were examined.

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Fig. 2. Photomicrographs of the FA (A and B, 8 weeks) and FP cartilage (C and D, 12 weeks) in the knee joint of rats. The FA cartilage was thicker in the SCI group than in the control group. The thickness at the FP region decreased and the subchondral bone penetrating into the cartilage was prominent. Noncartilaginous tissues (arrows). Toluidine blue staining (A–D). Immunohistochemical detection of the distribution of collagen type II in the FP cartilage (E and F, 10 weeks) and type I in the FA cartilage (G and H, 12 weeks). In the SCI cartilage, immunostaining to collagen type II showed moderate labeling in the superficial layer but slightly weak labeling from the middle to deep layer. Immunostaining to collagen type I showed intense labeling at the cartilage surface and the pericellular regions. Scale bars = 200 μm (A–F), 100 μm (G and H).
noncartilaginous tissues were rarely seen at the FA, TA, and TP regions, they were present at the FP region. The noncartilaginous mean thickness at the FP region of SCI knees reached 82.8 (62.1–91.0) μm at 4 weeks, 106.9 (55.7–135.8) μm at 10 weeks, and 124.1 (104.5–139.0) μm at 12 weeks.

At the FA region, we counted significantly fewer chondrocytes in SCI knees compared with that of control knees at all time points (4 weeks, 1666.7 [1555.6–1777.8] vs 2000.0 [1805.6–2555.6] per sq mm, power = 0.05; 8 weeks, 1388.9 [1222.2–1444.4] vs 2319.4 [2083.3–2500.0] per sq mm, power = 0.05; 10 weeks, 1541.7 [1166.7–1611.1] vs 2222.2 [1750.0–2444.4] per sq mm, power = 0.05; 12 weeks, 1541.7 [1444.4–1777.8] vs 2236.1 [1833.3–2416.7] per sq mm, power = 0.05). There was a statistically significant effect of time at the FA region of the SCI knee (P = 0.04), and the number of chondrocytes significantly decreased between 4 and 8 weeks and increased between 8 and 12 weeks for SCI knees. Also, at this region in SCI knees, there was an abundant number of lacunae without nuclei corresponding to chondrons interpreted as dead chondrocytes [clear “holes” within the dark background in Fig. 2(B)]. The FA cartilage matrix staining to toluidine blue of the SCI femur (FA and FP regions) showed no difference compared with that of control ones at any time point (P > 0.05). Cartilage matrix of SCI tibia stained significantly less than that of control at 8 weeks (TA region, 200.2 [196.2–201.7]) vs 207.6 [203.0–210.2] density units, power = 95.6; TP region, 194.8 [192.7–200.6] vs 203.2 [198.3–207.8] density units, power = 97.5; P < 0.05). Otherwise there was no difference between the groups (P > 0.05).

The cartilage matrix staining to toluidine blue of the SCI femur (FA and FP regions) showed no difference compared with that of control ones at any time point (P > 0.05). Cartilage matrix of SCI tibia stained significantly less than that of control at 8 weeks (TA region, 200.2 [196.2–201.7]) vs 207.6 [203.0–210.2] density units, power = 95.6; TP region, 194.8 [192.7–200.6] vs 203.2 [198.3–207.8] density units, power = 97.5; P < 0.05). The TA region matrix of SCI tibia also stained less than did that of control at 12 weeks (204.6 [197.4–207.8] vs 210.9 [209.6–218.8] density units, power = 80.3; P = 0.02). Time had no effect on the matrix density of the SCI knee joint (P > 0.05).

**Fig. 3.** Western blots of collagen type II (top) and type IX (bottom) in the articular cartilage of rat knee joints. Neither levels of collagen type II nor type IX presented any significant differences between the groups at all time points.

**Discussion**

The goal of this study was to identify the localization of cartilage alterations after SCI. Our results indicate that cartilage alterations after SCI exhibit the changes of the cartilage thickness, the decrease in the number of chondrocytes and the cartilage matrix staining, and the abnormality in the distribution of collagen type II and type I. These alterations differed between cartilage regions where the knee joint was placed in a flexed position.

One of the hallmarks of our findings was the changes in cartilage thickness. The mean cartilage thickness after SCI increased at unapposed regions (FA region) of the flexed knee joint and decreased at apposed regions (FP, TA, and TP regions). After unloading with immobilization, increased or decreased or unchanged thickness of the articular cartilage has been reported in various animal models. These contradictory results can be explained by a difference in immobilized positions (flexion or extension), animal species, or measurement regions. Knee joints of SCI rats are unloaded and restricted in flexion. Mechanical conditions in the knee cartilage of our rat models with SCI compared favorably with those from O’Connor. Therefore, to verify the influence of unloading and immobilization on the articular cartilage after SCI, we measured at four cartilage regions (FA, FP, TA, and TP regions) as described by her. She examined the knee cartilage of rat models of unloading combined with nonrigid immobilization in flexion and measured the cartilage thickness between the cartilage surface and the osteochondral junction. After 4 weeks, the cartilage thickness in the unloading combined with restricted movement increased by 15–22% at the FA region and by 10% at the TA region, although there were no changes at the FP and TP regions.

**LEVELS OF COLLAGEN**

The immunoblot of collagen type II and type IX in the combined articular cartilage from the femoral, tibial, and patellar cartilage is shown in Fig. 3. Although the signal for collagen type II and type IX antigens was detected in all samples, there were no significant differences when comparing control with SCI group and time effect among four cohorts (P > 0.05).
incongruent with these changes in the cartilage thickness by the suppression of mechanical forces. Based on the examination obtained by light microscopy, the thinning of knee joint cartilage was mostly implicated in the subchondral bone penetrating into the cartilage. Carter et al.17-19 postulated that the advancement of the subchondral bone front toward the joint surface is inhibited by intermittent hydrostatic pressure generated in the deep layers of cartilage during cyclic loading associated with weight bearing and transarticular muscular contractions. In the SCI rat, the hindlimb joints were unloaded, and muscular contractions related to spasticity (involuntary movements and agonist–antagonist co-contractions) different from normal contractions were present. Consequently, the inhibition may decrease at apposed regions and increase at unapposed regions, following a disorder of the inhibition by intermittent hydrostatic pressure. Vanwanseele et al.6,7 found the thinning of the human knee cartilage after SCI using three-dimensional magnetic resonance imaging. Although this thinning is compatible with those found in this study, we further identified the pathogenesis of the thinning and confirmed contrasting differences between the FA and FP regions.

We have shown that the number of chondrocytes decreases at the anterior regions, especially at the FA region (unapposed regions). In contrast, Thaxter et al.13 reported fewer chondrocytes at apposed regions of the flexed knee but not at unapposed regions after unloading with immobilization. Furthermore, they observed noncartilaginous tissues only within unapposed regions13. These results are not also in line with ours. Mechanical forces have great influence on the synthesis and rate of turnover of proteoglycans.4 Depletion of proteoglycan is one consistent feature of cartilage alterations observed in various animal models with the suppression of mechanical forces.2,10-12,14,16-18. After unloading with immobilization, reductions in the proteoglycan content at unapposed regions were more severe than those of apposed regions.2,16. However, in this study SCI diminished the proteoglycan content only at apposed regions (medial tibial condyle) but not at unapposed regions. In the femoral and tibial cartilage, immunolabeling to collagen type II was noted comparably in the superficial layer but noted weakly from the middle to deep layer, although no significant differences in the combined articular cartilage from the femoral, tibial, and patellar cartilage were detected by Western blot. This result of the immunohistochemical analysis conflicts with the changes obtained in osteoarthritis (a decrease in the superficial layer and a retention from the middle to deep layer).5. SCI patients develop occasionally osteoarthritis but may differ in nature. Finsterbusch and Friedman compared articular changes after peripheral denervation with those after immobilization, and found specific changes caused by nerveectomy; degeneration in the middle layers of cartilage, suggesting that nutritional deficiency was involved. Although this finding is similar to those observed in our study, it is currently unclear whether or not an influence through nerve tissues lies between our results. In the SCI cartilage, the pericellular regions in the superficial layer were labeled for collagen type I. This finding is indicative of the abnormality of extracellular matrix synthesis in chondrocytes5. Furthermore, the articular cartilage where collagen type I is excessively synthesized is vulnerable and leads to tissue destruction5.

Study limitations include small sample size and limited statistical power, precluding ruling out chance findings. Whether the results represent a random finding due to the small sample size or are due to, for example, the dragging load on the anterior knee after SCI remains open for discussion.

In conclusion, our findings exhibit properties characteristic of the articular cartilage after SCI. Although knee joints of SCI rats are unloaded and restricted in movement, our results are inconsistent with the cartilage alterations in the absence of mechanical forces. Cartilage alterations after SCI would not be explained by only a suppression of mechanical forces by unloading and immobilization, but there may be influences on the cartilage in addition to the change in mechanical forces.

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