Histological Evaluation of Lumbar Spine Changes in Rats with Collagen-induced Arthritis

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ABSTRACT
Background  To histologically evaluate lumbar involvement in rheumatoid arthritis (RA) by investigating rats with collagen-induced arthritis (CIA) and to assess the potential effects of RA on the discovertebral joints and facet joints.

Methods  Seven-month-old female Sprague-Dawley rats were divided into groups with CIA and without CIA (control). All rats were sacrificed at 8 weeks after initial sensitization and the lumbar spine (L5/6) was harvested. Then the lumbar spine block specimens were stained with Villaneuva bone stain and sectioned in the mid-sagittal plane. The left facet joints were also sectioned in the midaxial plane. Specimens were studied under a microscope and infiltration of inflammatory cells was investigated.

Results  In the CIA group, lumbar lesions were observed in 13/18 rats (76%). Lymphocytes infiltrated into the anterior rim of the vertebral bodies only in 2 rats, while lymphocytes infiltrated the facet joints only in 4 rats. Both sites were involved in 7 rats. In addition, osteoclasts invaded the anterior rim of the vertebral bodies and formed cavities that also contained lymphocytes. Formation of pannus was seen in the facet joints in 11/18 rats.

Conclusion  In CIA rats, infiltration of inflammatory cells into the anterior rim of the vertebral bodies alone or into the facet joints alone was demonstrated in 2 rats and 4 rats, respectively, while both sites were involved in 7 rats. Therefore, lesions at the anterior rim of the vertebral body did not arise secondary to facet joint involvement, but were caused by CIA along with synovial lesions of the facet joints.

Key words  collagen-induced arthritis rat; lumbar spine; rheumatoid arthritis

Rheumatoid arthritis (RA) is characterized by synovial inflammation, a profound lack of bone and cartilage repair in the peripheral joints. In patients with rheumatoid spondylitis, thoracic and lumbar lesions were once considered to be rare compared to cervical lesions such as vertical subluxation (VS), atlantoaxial subluxation (AAS), or subaxial subluxation (SAS). However, recent studies have revealed that RA involves the lumbar spine as well as the cervical spine, demonstrating abnormal findings on plain X-ray films of the lumbar spine in 57% of RA patients and lumbar endplate or facet erosion in 76.6% on magnetic resonance imaging (MRI).

RA often involves synovial joints of the cervical spine, including the atlanto-occipital joint, atlantoaxial joint, and facet joints. In the lumbar spine, erosion of discovertebral joints and facet joints has been reported in RA patients. However, there is no synovium in the discovertebral joints, so the pathology of lumbar spondylitis in RA patients remains unclear. In addition, histological evaluation of RA lesions in both the discovertebral joints and facet joints at the same spinal level has not yet been performed.

Collagen-induced arthritis (CIA) in rats is a well-known animal model of RA that shows clinical, histological, and immunological similarities to the human disease. CIA rats develop chronic synovitis, inflammatory cell infiltration, pannus formation, destruction of cartilage, and erosion of bone. These changes occur around 3 weeks after sensitization by injection of bovine type II collagen dissolved in Freund’s incomplete adjuvant.

In the present study, CIA rats were investigated to evaluate lumbar involvement in RA histologically and to determine the potential influence of RA on the discovertebral joints and facet joints.

MATERIALS AND METHODS
Animal model
Seven-month-old female Sprague-Dawley rats (retired breeder animals weighing 275-360 g; Shimizu Laboratory Supply, Kyoto, Japan) were used to create the model of adult human RA for this study. This experiment was conducted at the animal study facility of Tottori University Faculty of Medicine, and was approved by
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the Animal Experiment Ethical Committee of Tottori University. The approval number was 11-Y-60. The animals were given rat chow (CE-2, Clea, Tokyo, Japan; 1.18 g/100 g Ca, 250 IU/100 g vitamin D3) and tap water ad libitum, and were maintained at 24 °C with a 12-hour lighting time (7:00 AM to 7:00 PM). After 2 weeks of acclimation, the rats were randomly divided into 2 groups and used for the following experiments.

The CIA group (n = 20) received sensitization with collagen, while the control group (n = 5) received an injection of saline instead of collagen.

**Preparation of the CIA model**

Under intramuscular anesthesia with pentobarbital sodium (40–50 mg/kg of body weight), 1.0 mL of an emulsion containing 500 mg of bovine type II collagen in a 0.3% acetic acid solution (catalog no. K-41; Cosmo Bio, Tokyo, Japan) and 500 mg of Freund’s incomplete adjuvant (catalog no. 263910; Difco, Detroit, MI) was injected intracutaneously at 3 sites on the buttocks of each rat (CIA group). For additional sensitization, 0.5 mL of the same emulsion was injected at 2 sites on the buttocks 1 week after initial sensitization. In the control group, the same volume of saline was injected according to the same protocol as in the CIA group.

**Evaluation of arthritis**

Clinical evaluation of arthritis was performed by measuring the body weight and hind paw thickness before and 1, 3, and 8 weeks after initial sensitization. Hind paw thickness was evaluated by measuring the ankle width from the medial malleolus to the lateral malleolus using a constant-tension caliper. Hind paw thickness was expressed as the mean value for both hind limbs.

**Histological evaluation**

Eight weeks after initial sensitization, the rats were anesthetized by intramuscular injection of pentobarbital sodium (40–50 mg/kg of body weight) and sacrificed by cardiac puncture. The lumbar spine (L5/6) was harvested and stained with Villaneuva bone stain for 7 days, followed by dehydration in an ethanol series and embedding in methyl-methacrylate without decalcification. The resulting lumbar spine block specimens were sectioned in the midsagittal plane (Fig. 1). In addition, the left facet joint was sectioned in the midaxial plane (Fig. 2). Sections were cut at a thickness of 5 µm on a microtome (Leica Biosystems, Solms, Germany), and infiltration of osteoclasts or inflammatory cells like lymphocytes were investigated under a microscope.

Osteoclasts were defined as a large cell with multiple nuclei in the bone surface and lymphocytes were defined as an about 7 µm in diameter cell with dark stained nucleus.

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**Fig. 1.** Frontal view of the lumbar spine (L5/6). The blue dotted line indicates the midsagittal plane.

**Fig. 2.** Lateral view of the lumbar spine (L5/6). The white arrow indicates the facet joint. The blue dotted line indicates the midaxial plane through the facet joint (L5/6).
Statistical analysis
The Mann-Whitney \( U \) test was employed for statistical analysis using SPSS software (Dr. SPSS II for Windows Version 11.0.1J, SPSS, Tokyo, Japan). A probability value of less than 0.05 was considered significant.

RESULTS
In the CIA group, 2 rats died before 8 weeks and 18 rats were studied.

All of the rats in the control group survived.

Body weight
Body weight tended to increase gradually over time in the control group, while it decreased in the CIA group.

Table 1. Lumbar lesions with inflammatory cell infiltration in CIA rats

| Anterior rim of the vertebral body | Facet joint | Both sites | None |
|-----------------------------------|-------------|------------|------|
| n (%)                             | 2 (11%)     | 4 (22%)    | 7 (39%) | 5 (28%) |

CIA, collagen-induced arthritis.

Fig. 3. Histological features in CIA rats. Black frame showed the anterior rim of the vertebral body (A). In CIA rats, there was invasion of osteoclasts and formation of cavities containing lymphocytes in the anterior rim of the vertebral body (B1, B2). White arrows and black arrow indicated osteoclasts and lymphocytes, respectively. Villaneauva bone stain. CIA, collagen-induced arthritis.

At 1, 3, and 8 weeks after initial sensitization, mean body weight was respectively 316 g, 288 g, and 289 g in the CIA group versus 320 g, 322 g, and 328 g in control group. At 3 and 8 weeks, body weight was significantly lower in the CIA group than in the control group (\( P < 0.05 \)).

Development of arthritis
In the CIA group, the incidence of arthritis was 0%, 77%, and 94% at 1, 3 and 8 weeks after initial sensitization, respectively.

Hind paw thickness
At 1, 3, and 8 weeks after initial sensitization, mean hind paw thickness was respectively 6.1 mm, 8.3 mm, and 9.0 mm in the CIA group versus 6.0 mm, 6.1 mm, and 6.1 mm in the CIA group.
mm in the control group. At 3 and 8 weeks, hind paw thickness was significantly greater in the CIA group than the control group ($P < 0.05$).

**Histological features of arthritis**

In the CIA group, histological changes were detected at the anterior rim of the vertebral bodies (Fig. 3A) and in the facet joints. Lumbar lesions were observed in 13/18 rats (76%) (Table 1). Two rats had infiltration of lymphocytes into the anterior rim of the vertebral bodies alone, while infiltration into the facet joints alone was seen in 4 rats, and both sites were involved in 7 rats.

Osteoclasts or lymphocytes were found in the anterior rim of the vertebral bodies in 9/18 rats (Figs. 3B1 and B2). In CIA rats, examination of axial sections of the facet joints revealed pannus formation and cartilage erosion in these synovial joints in 11/18 rats (Figs. 4A1 and A2). There were no inflammatory cells in the intervertebral discs or at the posterior rim of the vertebral bodies in CIA rats. In control rats, no abnormalities were detected (Figs. 5A and B).

**DISCUSSION**

We histologically evaluated lumbar spine involvement in CIA rats as an animal model of RA, and we found inflammatory cell infiltration into the anterior rim of the vertebral bodies and into the facet joints. Osteoclasts also invaded the anterior rim of the vertebral bodies.

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**Fig. 4.** Histological features of the facet joints in CIA rats. It revealed that pannus formation and cartilage erosion in the facet joints (A1, A2). Villaneuva bone stain.

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**Fig. 5.** Histological features of the discovertebral joints and facet joints in control rats. No abnormalities were seen (A, B). A, B: Villaneuva bone stain.
and lymphocytes were induced from the bone marrow. Moreover, pannus was formed in the facet joints. These findings suggest that the anterior rim of the vertebral bodies may also be affected by RA, in addition to the synovial facet joints.

In RA patients, major pathological lesions of the cervical spine include VS, AAS, and SAS. Regarding VS and AAS, Reiter and Boden reported that the occiput-C1 and C1-C2 articulations were pure synovial joints, so these articulations could be primary targets for rheumatoid involvement. In VS, erosion of the occiput-C1 and C1-C2 joints reduces the vertical distance between the brain stem and the odontoid process. In AAS, destructive synovitis leads to ligamentous laxity and erosion of bone with resultant instability. Because the C1-C2 facets are oriented in the axial plane, there is no bony interlocking to prevent subluxation of this joint. In SAS, Ball reported that cervical instability and subluxation were associated with erosive arthritis of the facet joints. Although the prevalence of these conditions has not been determined recently, VS, AAS, and SAS were respectively reported in up to 38%, 49%, and 36% of RA patients before the introduction of biological agents.

It was previously thought that the lumbar spine was rarely involved by RA compared with the cervical spine. Lawrence and colleagues reported that only 5% of men and 3% of women with RA had lumbar spine involvement on plain X-ray films. However, Ibrahim et al. used MRI to reveal lesions of the cervical and lumbar spine in 42.3% and 56.2% of RA patients, respectively. They reported that lumbar spinal lesions were significantly associated with cervical lesions, but they did not discuss why the incidence of lumbar lesions was higher. In addition, Sakai et al. reported that 47 of 104 RA patients (45.2%) had abnormalities of the lumbar discov-vertebral joints on plain X-ray or MRI. They indicated that the initial RA lesions of lumbar spine were erosive or sclerotic changes at the anterior rim of the vertebral bodies, but they did not evaluate the facet joints.

Moreover, Yamada and colleagues detected erosion of the discovertebral joints and facet joints by MRI in 70.6% and 76.6% of RA patients, respectively. However, histological evaluation of both the discovertebral and facet joints at the same level has not been performed previously. In the present study, infiltration of inflammatory cells into the anterior rim of the vertebral bodies alone was seen in 2 CIA rats, while only the facet joints were affected in 4 animals and both sites were involved in 7. We also found osteoclasts invading the anterior rim of the vertebral bodies and induction of lymphocytes from the bone marrow. To observe the osteoclasts, tartratere-sistant acid phosphatase (TRAP) stain was more useful than Villanueva bone stain. However, we considered it was important to find lymphocytes in the eroded bone surface to reveal the inflammation at the discovertebral joint. For this condition, to maintain the structure of bone surface was needed. Therefore, our study did not adopt TRAP stain with decalcification, but Villanueva bone stain without decalcification.

In RA patients, some authors have also reported infiltration of osteoclasts and lymphocytes into inflammatory lesions of the limbs, and these changes are recognized as pathognomonic of RA. RA is characterized by synovial inflammation of peripheral joints. The facet joints are also synovial joints, and thus can be involved in RA. Since there is no synovial tissue at the anterior rim of the vertebral bodies, the reason why this region of the spine is affected by RA remains unclear. Heywood and Meyers studied patients with thoracic and lumbar rheumatoid spondylitis. They reported that erosion of the facet joints caused functional incompetence and intervertebral instability, leading to enthesopathy with inflammatory degeneration of collagen at the discovertebral joints. However, we found that inflammatory cells only infiltrated into the anterior rim of the vertebral bodies in 2 CIA rats or only infiltrated the facet joints in 4 rats, suggesting that these lesions of the vertebral bodies and facet joints arose independently. Some authors have reported that the vertebral endplate is also affected by RA since it is composed of hyaline cartilage with type II collagen. However, the anterior rim of the vertebral body does not contain the endplate, but is the site of the enthesis composed of ligament, fibrocartilage, and bone. There have been some necropsy reports of erosive lesions at the anterior entheses of the lumbar vertebral bodies in RA patients. Therefore, it seems possible that primary enthesopathy occurs at the anterior rim of the lumbar vertebral bodies, rather than these changes being secondary to facet joint involvement.

There were several limitations of this study. First, we did not observe long-term changes of the lumbar spine. In CIA rats, inflammation subsides after 6 to 8 weeks and then the healing process starts. Also, inflammation of the spine develops later than that of the peripheral joints in CIA animals. Therefore, it is difficult to find changes mimicking those of the lumbar spine in chronic RA. Second, we did not perform MRI, which was reported to be effective for evaluation of rheumatoid spondylitis in humans. However, it is difficult to detect evidence of inflammation on MRI in small animals such as rats. Third, lumbar lesions might well differ between quadrupedal rats and bipedal humans. Our study revealed that the anterior rim of the vertebral body is also targeted by inflammation in CIA, as well as the synovium of the facet joints.
joints of bipedal humans are subjected to greater loads than those of quadrupedal rats, especially if facet joint involvement leads to functional incompetence, discovertebral lesions of the lumbar spine might be more severe in RA patients. Fourth, we evaluated the specimens in only one section. Therefore, there is a possibility that inflammatory cells existed in the specimens that we decided there was no inflammatory cell. Fifth, we did not evaluate cervical spine in CIA rats. RA often involves synovial joints of the cervical spine, therefore, we should compare typical cervical lesions in CIA rats with lumbar lesions in it.

In conclusion, infiltration of inflammatory cells was confined to the anterior rim of the vertebral bodies or only affected the facet joints in some CIA rats, while both sites were involved in other animals. This suggests that lesions at the anterior rim of the vertebral bodies were not secondary to functional incompetence of the facet joints, and that this site was targeted by CIA along with the synovium of the facet joints.

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