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Nociceptive phenotype alterations of dorsal root ganglia neurons innervating the subchondral bone in osteoarthritic rat knee joints

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SUMMARY

Objective: Subchondral bone plays a role in generating knee joint pain in osteoarthritis (OA). The objective of this study was to clarify nociceptive phenotype alterations of subchondral bone afferents of the distal femur in mono-iodoacetate (MIA)-induced OA rats.
Methods: OA was induced by intra-articular injection of MIA in rats. Two different retrograde tracers were separately injected into the knee joint cavity and the subchondral bone to identify joint and subchondral bone afferents. Immunohistochemistry was used at 2 weeks (early stage) and 6 weeks (advanced stage) after MIA injection to determine the expression of nociceptive markers (calcitonin gene-related peptide (CGRP) and tyrosine receptor kinase A (TrkA)) and the soma size distribution of CGRP-immunoreactive (IR) neurons. Histological subchondral bone and cartilage damage was scored according to the Osteoarthritis Research Society International grading system. Pain-related behavior was evaluated using weight distribution and mechanical sensitivity of the hind paw.
Results: OA caused an up-regulation of CGRP, TrkA and enlargement of soma size of CGRP-IR neurons in both joint and subchondral bone afferents. CGRP and TrkA expression in subchondral bone afferents gradually increased over 6 weeks. Furthermore, up-regulation of CGRP and TrkA in subchondral bone afferents displayed a strong correlation with the subchondral bone damage score.
Conclusion: Up-regulation of nociceptive markers in subchondral bone afferents correlated with subchondral bone damage, suggesting that subchondral bone is a therapeutic target, especially in the case of advanced stage knee OA. In particular, CGRP and TrkA are potentially molecular therapeutic targets to treat joint pain associated with subchondral lesions.
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Introduction

Knee pain is a major source of disability and reason for hospital visits in patients with knee osteoarthritis (OA)[1]. Although the mechanism of OA progression has been well documented, pain pathophysiology is largely unknown. Recent accumulating clinical evidence[2–7] indicates that subchondral bone plays a role in generating joint pain in OA. Subchondral bone marrow lesions (BMLs) detected on magnetic resonance imaging (MRI) in knee OA are strongly associated with intense pain[2–5]. Bone attrition, which is defined as a flattening or depression of the subchondral bone by using x-rays or MRI, is strongly associated with the presence of pain[6,7].

The cell bodies of primary sensory neurons innervating subchondral bone are located in the dorsal root ganglia (DRG). The calcitonin gene-related peptide-immunoreactive (CGRP-IR) DRG neurons are regulated by nerve growth factor (NGF) and considered highly sensitive to inflammation[8]. A previous study showed that more than 90% of CGRP-IR DRG neurons were immunostained for the high-affinity NGF receptor tyrosine receptor kinase A (TrkA)[9]. Our previous study[10] showed that the majority of DRG neurons innervating the subchondral bone in normal rat knee joints were CGRP- and TrkA-IR, implying that the subchondral bone is highly sensitive to nociceptive stimuli and inflammation. However, nociceptive phenotype alterations of DRG neurons innervating the subchondral bone in knee OA have not been clarified.

It has been reported that mono-sodium iodoacetate (MIA) injection into the rat knee joint disrupts chondrocyte metabolism,

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leading to cell death and subsequent loss of articular cartilage with subchondral bone changes[11,12]. The joint damage observed in the MIA model is similar to the joint damage observed in OA[13,14]. The model demonstrates a clear interrelationship between cartilage damage and subchondral bone changes and could be used to study the role of subchondral bone in the progression of OA[14].

The objective of this study was to clarify nociceptive phenotype alterations of DRG neurons innervating the subchondral bone of the distal femur in MIA-induced OA rat. Specifically, we evaluated the association of nociceptive phenotype alterations with histological damage in the subchondral bone. Two different retrograde tracers were separately injected into the knee joint cavity and the subchondral bone to identify joint and subchondral bone afferents. Immunohistochemistry was used at 2 weeks (early stage) and 6 weeks (advanced stage) after MIA injection to determine the expression of nociceptive markers (CGRP and TrkA) and the soma size distribution of CGRP-IR neurons.

Materials and methods

Animals

Male Sprague–Dawley rats (6 weeks old, weight 250–300 g) were used in this study. A preliminary study confirmed that the distal femoral epiphysis was completely separated from the diaphysis by the epiphyseal plate in 6-week-old rats. The rats were divided into three groups: 2 weeks, 6 weeks after MIA injection and control (saline injection). Thirty-six rats were used for immunohistochemistry studies, eighteen rats used for histologic studies and twelve rats used for behavioral studies. All experiments were approved by the Animal Care and Use Committee of Kochi University. All outcome measurements were made by an observer blinded to treatment.

Induction of OA

After being anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal), rats were injected with 3 mg of MIA in 25 μl of saline (Sigma–Aldrich, St. Louis, MO) or 25 μl of saline (control group), using a 27 G needle with a Hamilton syringe inserted through the patellar ligament into the intra-articular space of the left knee.

Retrograde labeling

At 2 or 6 weeks after MIA or saline injection, all rats were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal). After shaving, a 20 mm skin incision was made over the lateral side of the left knee. For retrograde labeling, 1.5 μl FB (10 mg/ml in saline; Polysciences, Inc. Warrington, PA) was injected into the left distal femoral epiphyses, while 10 μl DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes) (5 mg/mL in N, N dimethylformamide) was injected into the left knee joints. Both tracers were injected into the same joint. A 27 G needle with a Hamilton syringe was used to inject the retrograde tracers. FB injection into the distal femoral epiphysis was performed by drilling a 1 mm hole through the cortical bone on the lateral side of the femoral epiphysis. The FB diffusion can be confirmed with a hand-held UV illumination device (365 nm wavelength). A preliminary study confirmed that FB or black ink had permeated the medial and lateral condyle of the distal femur without leakage to the intraarticular and diaphysis similarly among rats with OA (2 weeks and 6 weeks after MIA injection) and control (n = 6 for each group) (also see Supplementary Fig. 1). Drill holes were sealed with cyanoacrylate to prevent tracer leakage into the surrounding tissues. The injection sites were inspected for tracer leakage using the hand-held UV illumination device and washed with phosphate-buffered saline (PBS). Knees that showed evidence of tracer leakage to the surrounding tissues were excluded from this study. DiI injection was performed through the patellar ligament into the intra-articular space of the knee after placement of the FB. After DiI injection, the wound was closed with 4-0 nylon. At 14 days after FB and DiI injection, animals were euthanized with an overdose of sodium pentobarbital (150 mg/kg, intraperitoneal), and the left lumbar DRGs (L3, L4) were obtained. The DRGs were placed in 4% paraformaldehyde for 2 h and 30% sucrose overnight, embedded in OCT compound (Sakura Finetek, Torrace, CA, USA) and frozen in −80°C until sectioning. Fourteen-micrometer frozen sections were then cut using a cryostat. Sections were mounted on slides using Fro-Tissuer Pen (Agar Scientific, Stansted, UK).

Immunohistochemistry of DRGs

Alterations in the primary DRG neurons were evaluated using the expression of CGRP and TrkA. Since the majority of neurons innervating the subchondral bone of the distal femur were localized in L3 DRGs and neurons innervating the knee joint were localized mainly in L3 and L4[10], L3 and L4 DRGs were used for immunofluorescence reactions for CGRP and TrkA. The sections incubated in rabbit anti-CGRP antibody (1:1000 T-4032; Peninsula Laboratories, Belmont, CA) and goat anti-TrkA antibody (1:200 AF1056; R&D System, Minneapolis, MN) overnight in a humid chamber in the fridge. The next day, secondary detection was performed with goat anti-rabbit IgG-FITC (1:500 sc-2012; Santa Cruz Biotechnology, Inc. CA) for CGRP and rabbit anti-goat IgG-FITC (1:500 sc-2777; Santa Cruz Biotechnology, Inc. CA) for TrkA. All antisera were diluted in PBS containing 1% normal goat serum for CGRP and 2% rabbit serum for TrkA. Before, between, and after each incubation step, the sections were washed three times for 5 min in PBS. As a negative control, representative sections were processed without a primary antibody. Finally, all sections were mounted with Vectashield (Vector, Burlingame, CA).

Sections were viewed with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Representative photos of DRGs were taken using a confocal microscope Olympus FV-1000D/IX81 (Olympus, Tokyo, Japan). At least 50 sections were cut from each DRG, and FB- and DiI-labeled neurons with visible nuclei were counted from every fifth section to eliminate the possibility of double counting. Because L3 and L4 DRGs were used for immunofluorescence reactions, a total of at least 100 sections of DRGs were cut from each rat and at least 20 sections were evaluated.

For each FB- and DiI-labeled neuron, CGRP and TrkA were quantified as the percentage of total FB- and DiI-labeled neurons. Data were presented as the median and 95% confidence interval (CI). We compared the percentage of CGRP- and TrkA-IR neurons in FB- and DiI-labeled neurons at 2 weeks, 6 weeks after MIA injection and control ($n = 6$ rats for each group).

The soma size of FB- and DiI-labeled CGRP-IR neurons was examined. According to the measured area, neurons were classified as small (<600 μm^2), medium (600–1200 μm^2), and large (>1200 μm^2)[15–17]. We compared the soma size of FB- and DiI-labeled CGRP-IR neurons at 2 weeks, 6 weeks after MIA injection and control ($n = 6$ rats for each group).

Histological evaluation of knee joint

Knee joint histology with special reference to subchondral bone was evaluated at 2 weeks, 6 weeks after MIA injection and control. The knee joints were placed in 10% formalin for 3 days,

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