

Nociceptive sensory innervation of the posterior cruciate ligament in osteoarthritic knees

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Abstract

Introduction Although the posterior cruciate ligament (PCL) is considered to contain not only proprioceptive but also nociceptive sensory fibers, there is a lack of information about nociceptive sensory innervation of the PCL. We hypothesized that the PCL has constant nociceptive sensory innervation, suggesting the possible source of osteoarthritic (OA) knee pain.

Materials and methods Innervation of the PCL was examined by immunohistochemistry with particular reference to nociceptive nerve fibers in OA knees. Sensory nerve fibers were semi-quantitatively counted in the PCL of OA knees, comparing with non-OA knees. Protein gene product 9.5 (PGP9.5) as a general neuronal marker and calcitonin gene related peptide (CGRP) as a marker for nociceptive neuron were used.

Results The PCLs had constant CGRP-immunoreactive (IR) nerve fibers in both OA and non-OA knees. The difference of the CGRP-IR nerve density between groups did not reach a statistical significance ($p = 0.062$). For PGP9.5-IR nerve fibers, however, the PCLs in OA knees were statistically less innervated than non-OA knees ($p = 0.0009$).

Conclusions Our results showed that, in spite of a significant decrease in total innervation in OA knees, the PCLs have constant nociceptive sensory innervation. Although

the relationship between the decrease in total innervations in the PCL and OA pathophysiology is still unclear, the PCL is the possible source of OA knee pain. Our results should be taken into account when examining the pain source of the OA knees and handling the PCL during total knee arthroplasty.

Keywords Posterior cruciate ligament · Knee · Osteoarthritis · Immunohistochemistry · Pain · Calcitonin gene related peptide

Introduction

The posterior cruciate ligament (PCL), the strongest ligament in the knee joint, is considered to have sensory function as well as stabilizing function. Historically, morphological studies using nonspecific staining, such as gold chloride, silver, and Masson's trichrome methods, confirmed the presence of neural network within the PCL [1–3]. Recently, nerve-specific staining methods used in a few immunohistochemical studies [4, 5] revalidated this observation. However, most previous studies paid attention only to the low-threshold mechanoreceptors [1, 2, 5, 6], which play a significant role in the proprioceptive function of the knee joint. To date, there is a lack of information about nociceptive sensory innervation of the PCL. We hypothesized that the PCL has constant nociceptive sensory innervation, suggesting the possible source of OA knee pain. We semi-quantitatively counted the sensory nerve fibers of the PCL taken from OA knees (OA-PCL) and non-OA knees (N-PCL) with immunohistochemical methods. We used protein gene product 9.5 (PGP9.5) as a general neuronal marker and calcitonin gene related peptide (CGRP) as a marker for nociceptive neuron.

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Materials and methods

All samples were obtained from patients during total knee arthroplasty (TKA) or anterior cruciate ligament (ACL) reconstruction. The OA-PCLs were from 10 patients with OA knees with a mean age of 77 years (70–85) and the N-PCLs were from 5 patients with ACL rupture with a mean age of 26 years (16–42). The severity of pain in patients with OA knees was evaluated using 100 mm visual analogue scale, preoperatively. The mean walking knee pain was 59 mm (range 36–100 mm). On the other hand, none of the patients with ACL rupture complained of knee pain. Two PCL samples for each patient, approximately 4 mm², were taken with 3-mm forceps, one from its femoral insertion and the other from middle of the PCL. As a positive control for immunohistochemistry test, inflamed synovium was also obtained from patients with OA knees. Ethics committee approval from our institution was obtained prior to the study. Each patient was informed of the objectives and risks of the study and gave his or her consent. The study was conducted in compliance with the Declaration of Helsinki.

Immunohistochemistry

All samples were fixed in 4% paraformaldehyde for 24 h, placed in 30% sucrose for another 24 h, embedded in OCT compound (Sakura Finetek, Torrance, California), rapidly frozen, and kept at -80°C until sectioning. Fourteen micrometer frozen sections were then cut using a cryostat. Each section was observed with a microscope before staining to confirm the representative section.

The sections were soaked in methanol containing 0.3% H₂O₂ for 10 min to quench endogenous peroxidase, blocked in 3% normal goat serum for 30 min, and then incubated with rabbit anti-human PGP9.5 antibody (Ultraclone, 1:1,000) or rabbit anti-human CGRP antibody (Peninsula T-4239 1:1,000) overnight at room temperature. The next day, the sections were incubated with biotinylated goat anti-rabbit IgG diluted 1:200 in PBS containing 0.05% Triton X-100 and 1% normal goat serum for 30 min at room temperature. The sites of antibody binding were visualized using avidin–biotin peroxidase method (ABC kit, Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:200 for 30 min at room temperature. Finally, the sections were treated with 3,3'-Diaminobenzidine for 3 min, counterstained with hematoxylin, and mounted on slides. Before, between, and after each incubation step, the sections were washed three times for 5 min in PBS. Negative controls were made by substituting primary antibody with non-immune rabbit serum.

Semi-quantitative analysis

Each section was observed with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany). The CGRP- and PGP9.5-immunoreactive (IR) nerve fibers were counted in randomly selected five sections for each sample at a magnification of 200 \times . Only nerve fibers longer than 30 μm in length were counted. The area of each observed section was measured using Image J software (National Institutes of Health: <http://rsb.info.nih.gov/nih-image/>) and the nerve density was expressed as nerve fibers/mm².

Statistical analysis

All values were expressed as median (interquartile range). For comparison of the nerve density within one group and between groups, Mann–Whitney *U* test was used (SPSS version 16.0, SPSS Inc, Chicago, USA). A $p < 0.05$ was considered statistically significant.

Results

In general, both CGRP- and PGP9.5-IR nerve fibers were consistently observed in all samples with little nonspecific staining (Fig. 1). The harvested PCLs included a small amount of richly vascularized and innervated epiligament (approximately less than 1/10 of the total area). Nerve fibers were found predominantly in the epiligament in a perivascular distribution. In the deep substance of the ligament, less blood vessels and nerve fibers were found than in the epiligament.

The nerve density was higher at the femoral insertion site than the middle part of the PCL for CGRP- and PGP9.5-IR nerve fibers. The OA-PCLs showed various degenerative changes such as loose collagen fibers, mucomyxoid degeneration, and disorganized collagen architecture with some disruption.

The OA-PCLs tended to have less innervation than the N-PCLs. However, the difference of the CGRP-IR nerve density between the OA-PCLs and the N-PCLs did not reach a statistical significance [0.140 (0.120, 0.153) vs. 0.155 (0.140, 0.183) fibers/mm²; $p = 0.062$] (Fig. 2a). For PGP9.5-IR nerve fibers, the OA-PCLs were statistically less innervated than the N-PCLs [0.230 (0.208, 0.295) vs. 0.375 (0.348, 0.450) fibers/mm²; $p = 0.0009$] (Fig. 2b). The ratio of the density of CGRP-IR to PGP9.5-IR nerve fibers (% CGRP) was 59 (45, 71)% in the OA-PCLs and 45 (34, 48)% in the N-PCLs. The OA-PCLs had significantly higher % CGRP than the N-PCLs ($p = 0.031$).

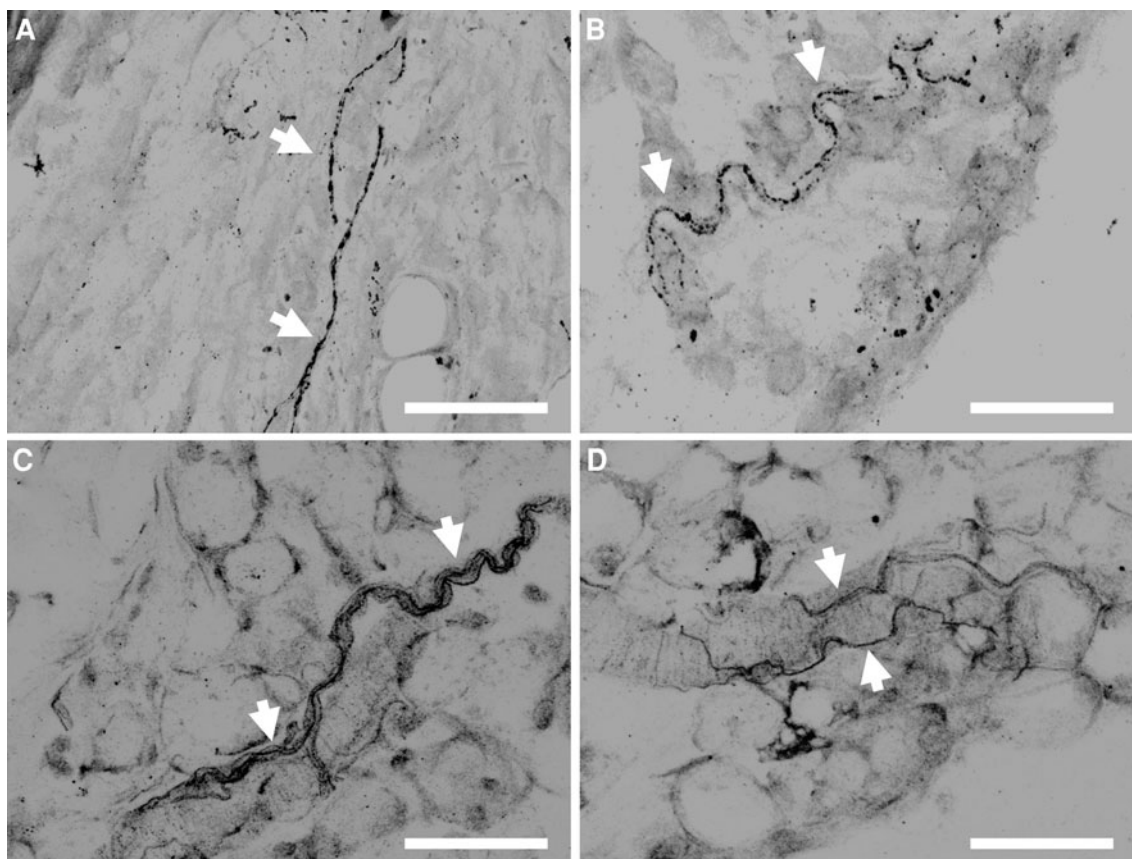


Fig. 1 Nerve fibers immunoreactive for calcitonin gene related peptide (a, b) and protein gene product 9.5 (c, d). Bar = 100 μ m

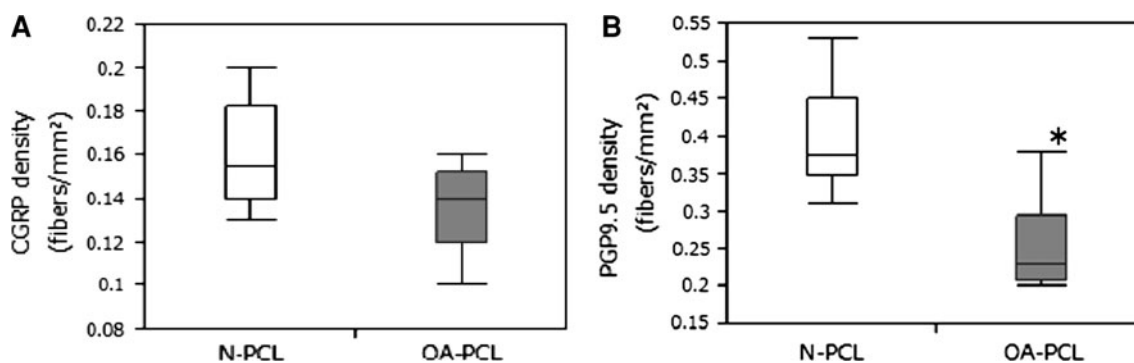


Fig. 2 Comparison of CGRP density (a) and PGP9.5 density (b) between OA-PCL and N-PCL. Values are represented as mean \pm SD. * $p < 0.05$ versus N-PCL

Discussion

Proprioceptive function of the knee joint is reported to decline with aging [7–10] and certain joint diseases [8, 9, 11]. The decrease in number of mechanoreceptors within ligaments, reported in animals [12] and humans [2, 6, 13], is presumably one of the underlying mechanisms. In addition, total number of knee joint afferents decreases with aging [14] and OA changes [15, 16] in rodents. Taken together, the decrease in the number of mechano-

receptors at least partially accounts for the age-related loss of joint innervation and progression of OA changes. Our results were consistent with this notion. The number of PGP 9.5-IR fibers in the OA-PCL was significantly smaller than the N-PCL. Anti-PGP 9.5 labels all neurons, including sympathetic and motor neurons as well as sensory neurons [17]. Ligaments, like many other connective tissues, are innervated with both sensory and sympathetic nerves [18]. Therefore, our results showed that, comparing with the normal knees, there was a

significant decrease in total innervation of the PCL including a subpopulation of proprioceptive and sympathetic nerves in the OA knees. Because peripheral nerves within the ligament play an essential role in homeostasis of ligaments and joints [19, 20], the decrease in total innervations can be detrimental to the structural integrity of ligaments and joints.

Although the issue of PCL retention versus sacrifice during TKA has been discussed largely over the past 20 years, there is, so far, no solid conclusion in this regard [21]. It is clear that the PCL plays an important role in the kinematics of the normal knee joint because of its biomechanical and proprioceptive functions. Therefore, it would be reasonable to retain the PCL during TKA if its functions were expected to be normal. However, all these functions of the PCL are doubtful in OA knees [4, 8]. As mentioned above, our results support previous reports regarding loss of the proprioceptive function of the PCL in OA knees. Furthermore, our results showed an interesting finding that the difference of the CGRP-IR nerve density between OA-PCL and N-PCL was not statistically significant. CGRP is a marker of somatic sensory neurons, typically responsible for nociceptive fibers [22]. Therefore, our results indicate that nociceptive inputs selectively remain in the PCL of OA knees, while the total innervation decreases. One possible reason for this finding is that OA and arthritic changes in knee cause an up-regulation of CGRP in rodent dorsal root ganglia [16, 23]. These findings will give new insights to the PCL issue in TKA and should be taken into account.

There are several limitations in this study. First, the N-PCLs might not be representative of normal PCL because they were obtained from chronic ACL-deficient knees. Although the N-PCLs were obtained more than 3 months after the ACL injury, there could have been injury- or instability-related changes of innervation within the PCL. Second, the N-PCLs were obtained from younger patients than the OA-PCLs. Not only OA changes but also aging presumably affected the differences of innervation between two groups. Third, there were more nerve fibers in the epiligament than the ligament substance. Although the area of the epiligament in each section was relatively constant, the counting results could have been affected by the way of sampling the tissue.

In conclusion, the PCL has constant nociceptive sensory innervation even in OA knees. Although the relationship between the decrease in total innervations in the PCL and OA pathophysiology is still unclear, the PCL is the possible source of OA knee pain. Our results should be taken into account when examining the pain source of the OA knees and handling the PCL during TKA.

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