A novel rat model of hip pain by intra-articular injection of nerve growth factor – characteristics of sensory innervation and inflammatory arthritis

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Abstract

Objectives. To determine the direct effects of intra-articular injection of nerve growth factor (NGF) into normal rat hips and the time course of pain-related mediator appearance.

Methods. Using 36 numbers of 8-week-old male Sprague–Dawley rats, 30 µl of 1% Fluoro-Gold solution (FG) (Sham-operated group; n = 12), 30 µl of 1% FG with 50 µg/ml NGF (NGF50 group; n = 12), and 30 µl of 1% FG with 100 µg/ml NGF (NGF100 group; n = 12) were injected into the left hip joints. Neurons in the dorsal root ganglion (DRG) labeled with FG, and FG and calcitonin gene-related peptide-immunoreactivity (CGRP-IR) were counted. The synovia in the left hip joint was examined histologically.

Results. The NGF50 and NGF100 groups showed evidence of synovitis without cartilage degeneration compared with the Sham-operated group. At 7 days, the proportions of CGRP-IR FG-labeled neurons were 12%, 18%, and 36% in the Sham-operated, NGF50, and NGF100 groups, respectively. At 14 days, the proportions were 13%, 22%, and 35% in the Sham-operated, NGF50, and NGF100 groups, respectively. At 7 and 14 days, the NGF50 and NGF100 groups showed a significantly higher proportion of CGRP-IR FG-labeled neurons than the Sham-operated group.

Conclusions. Intra-articular administration of NGF into the hip joint produces a novel rat model for hip pain.

Introduction

Pain originating from the hip has been classically described as presenting in the groin. However, referred pain may occur in osteoarthritis (OA) [1–2], osteonecrosis [3–4], acetabular labral lesion [5], and other hip disorders. Pain has been reported to affect the thigh or lower leg as well as the hip itself. A recent study of OA of the hip found that referred pain was localized to the groin (89%), buttock (38%), greater trochanter (27%), anterior thigh (33%), knee (29%), and lower back (17%), corresponding to the L1 to L5 dermatomes [6]. Thus, it is often difficult to distinguish between pain referred as from the hip and radicular pain originating from a lumbar lesion [7].

It is not clear why the symptoms and pain of inflammatory or degenerative processes are so varied in the area of the hip. Birnbaum et al. [8] described the hip as innervated by the obturator, femoral, sciatic, and superior gluteal nerves. Previously, we reported that dorsal root ganglion (DRG) neurons innervating the hip were distributed on multiple levels (L1–L4) [9] and that there is a difference in the level of innervation between the hip and inguinal skin [10]. Moreover, DRG neurons that innervate the hip and inguinal skin may overlap [11]. Animal models of hip pain are essential to clarify the pathogenesis of hip pain. However, such hip pain model has not been established so far.

Pain is transmitted to the dorsal horn of the spinal cord by small dark cells in the DRG, and these small DRG neurons are divided into nerve growth factor (NGF)-sensitive neurons and glial cell line-derived neurotrophic factor (GDNF)-sensitive neurons. NGF and GDNF regulate the expression of various pain-related molecules, including substance P (SP) and calcitonin gene-related peptide (CGRP) [12]. NGF-sensitive neurons express two types of the receptors, i.e., the high-affinity NGF receptor tyrosine kinase A and the low-affinity NGF receptor p75 [13]. Pain is mainly transmitted by CGRP-immunoreactivity (IR) neurons [9,14]. Previous studies have suggested that anti-NGF antibodies have analgesic effects on pathological pain [13]. However, these studies targeted neuropathic pain or pain from cutaneous tissue, and inflammatory hip pain has been unknown.

The purpose of this study was to determine the direct effects of intra-articular injection of NGF into normal rat hips and the time course of pain-related mediator appearance, focusing particularly on both inflammatory and neuropathic pain-related states using local tissues and sensory innervation of the peripheral nervous system including the DRG.

Materials and methods

We used 36 numbers of 8-week-old male Sprague–Dawley rats weighing 250–300 g. The protocols for the animal procedures followed in these experiments followed the 1996 revision of the National...
Institutes of Health guidelines for the Care and Use of Laboratory Animals and received approval from the ethics committee of our institution. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and were treated aseptically throughout the experiments. Using a 26-gauge needle, the following solutions were injected into the left hip of each rat: 30 μl of 1% Fluoro-Gold solution (FG: Fluorochrome, Denver, Colorado) in the Sham-operated group rats (n = 12), 30 μl of 1% FG with 50 μg/ml of NGF (recombinant human β-NGF, 256-GF100; R&D Systems, Minneapolis, MN) in the NGF50 group rats (n = 12), and 30 μl of 1% FG with 100 μg/ml of NGF in the NGF100 group rats (n = 12). The injected fluid was restricted to the joint cavity.

Seven days later, six out of twelve rats in each group were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and perfused transcardially with 0.9% saline, followed by 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The left hips were exposed and the soft tissues around the joint and cartilage including the synovium and capsule were resected (n = 6, each). Then DRGs between T13 and L6 on the left side were also resected. The resected limbs were prepared for histopathology and the DRG specimens were prepared for immunohistology. At 14 days, the remaining six rats in each group were processed in the same manner.

**Histopathology of the hip joint**

The resected limbs were cut at the pelvis and at the midfemur and immersed in buffered paraformaldehyde fixative at 4°C for 1 week. The specimens were demineralized in 10% EDTA for 2 weeks following by standard histological techniques using paraffin blocks for subsequent coronal sectioning. The samples were serially sectioned in steps of 8 μm, stained using hematoxylin and eosin (HE) used for principal method of pathological change, Safranin O and toluidine-blue staining used for the detection of cartilage. They were assessed by light microscopy using the synovitis score of Krenn et al. [15], which consisted of enlargement of the synovial lining cell layer (0–3 points), density of the resident cells (0–3 point), and inflammatory infiltrate (0–3 points). In total, 0–1 point was classified as no synovitis, 2–4 points were low-grade synovitis, and 5–9 points were high-grade synovitis. Cartilage change was graded as by Mankin score [16], consisted of structure (0–6 points), tidemark integrity (0–3 points), and density of the resident cells (0–3 points), Safranin O staining (0–4 points), and inflammatory infiltrate (0–3 points). In total, 0−1 3 points are classified as slight degeneration, 4−7 points are moderate, and 8−14 points are severe.

**Calcitonin gene-related peptide immunohistochemistry of DRG**

The specimens were immersed in the phosphate-buffered paraformaldehyde fixative overnight at 4°C. After storage in 0.01 M phosphate-buffered saline (PBS) containing 20% sucrose for 20 hours at 4°C, each DRG was sectioned at 12 μm on a cryostat and mounted on poly-l-lysine-coated slides. Endogenous tissue peroxidase activity was quenched by soaking the sections for 30 minutes in 0.3% hydrogen peroxide solution in 0.01 M PBS. The specimens were then treated in a blocking solution, 0.01 M PBS containing 0.3% Triton X-100 and 3% skimmed milk, overnight at 4°C. They were processed for CGRP immunohistochemistry using rabbit antibody to CGRP (1:1000; Chemicon), diluted with a blocking solution, and incubated overnight at 4°C. After incubation with labeled antibody, the sections were incubated with goat anti-rabbit Alexa 488 for CGRP-IR (1:1000; Molecular Probes, Eugene, Oregon). After each step, the sections were rinsed in 0.01 M PBS three times and studied using fluorescence microscopy.

The numbers of FG-labeled and CGRP-IR FG-labeled neurons were counted (Figure 1).

**Statistical analyses**

Differences between the groups in the number of FG-labeled DRG neurons in the DRGs between T13 and L6 were evaluated using a Tukey–Kramer test. The proportions of CGRP-IR FG-labeled neurons in the DRGs were compared using a Mann–Whitney U test followed by a Bonferroni correction. P < 0.05 was considered to be statistically significant.

**Results**

**Histopathology of the hip joint**

All the hips in both NGF 50 and NGF 100 groups showed inflammatory change with low-grade synovitis and angiogenesis in day 7. Synovitis score of each group was shown in Table 1. The histological features of synovitis were a single layer of lining cells, slightly increased cellularity, and floc, mostly perivascular situated lymphocytes or plasma cells. However, the hips did not show osteoarthritis-like appearances such as cartilage loss and degeneration, subchondral bone collapse, or subchondral cystic structure with Safranin O and toluidine blue staining. The NGF 50 and NGF 100 groups showed similar appearances (Figures 2 and 3). Sham-operated hips showed an expected normal appearance (Figure 4). Inflammatory change persisted in both the NGF 50 and NGF 100 groups until at least day 14. By contrast, osteoarthritic

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<th>Sham-operated group</th>
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<td>Enlargement of the synovial lining cell layer</td>
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<td>Density of the resident cells</td>
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<td>Inflammatory infiltrate</td>
<td>0.2</td>
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<td>Total</td>
<td>0.3*</td>
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Mann–Whitney’s U test followed by Bonferroni correction, *p = 0.0074, †p = 0.0049.
Figure 2. Histopathology of the hip joint in a NGF50 rat at day 7. A) Hematoxylin and eosin staining, B) Safranin O staining, and C) Toluidine-blue staining of 40 magnification (Scale bars are 500 µm). Each shows mild synovitis and angiogenesis (white arrow head) without cartilage degeneration in the femoral head (white arrow) and the acetabulum (black arrow head). D) Hematoxylin and eosin staining of 400 magnification (Scale bars are 50 µm) shows synovitis and angiogenesis (black arrow).

changes were not seen in any groups. Mean Mankin score was 0.3 in Sham-operated group, 0.7 in NGF50 group, and 0.7 in NGF100 group without significant differences (Sham-operated group versus NGF50, P = 0.522; Sham-operated group versus NGF100, P = 0.522; NGF50 versus NGF100, P = 1.000; respectively, Mann–Whitney U test followed by a Bonferroni correction).

Figure 3. Histopathology of the hip joint in a NGF100 rat at day 7. A) Hematoxylin and eosin staining, B) Safranin O staining, and C) Toluidine-blue staining of 40 magnification (Scale bars are 500 µm). Each shows mild synovitis and angiogenesis (white arrow head) without cartilage degeneration in the femoral head (white arrow) and the acetabulum (black arrow head). D) Hematoxylin and eosin staining of 400 magnification (Scale bars are 50 µm) shows synovitis and angiogenesis (black arrow).
Fluoro-Gold-labeled neurons in dorsal-root ganglia

Neurons with FG-labeling, in which FG was transported from the hip, were distributed in the left DRGs between T13 and L6 at day 7 (Figure 5). The labeled neurons were mainly found in the L3 and L4 DRG, and the proportion of FG-labeled neurons in the L1, L2, L3, and L4 DRGs was significantly higher than those in the L6 DRG (Tukey–Kramer test, \( P < 0.05 \)). Very similar distributions were observed in each group (data not shown). Furthermore, the proportions of FG-labeled neurons were not significantly different for each level of DRG among these groups.

The distributions and the proportions of FG-labeled neurons in DRG were not significantly different on day 7 and day 14 among the three groups.

Fluoro-Gold-labeled calcitonin gene-related peptide-immunoreactive neurons

FG-labeled CGRP-IR neurons were mainly present in the left DRGs between L1 and L4. Of the FG-labeled neurons on day 7, the mean proportion of CGRP-IR FG-labeled neurons in DRGs was 11.5% in the Sham-operated group, 18.1% in the NGF50 group, and 36.2% in the NGF100 group. The proportion increased dose dependently (Sham versus NGF50, \( P = 0.0084 \); NGF50 versus NGF100, \( P = 0.0007 \); NGF100 versus Sham, \( P = 0.0001 \); respectively, Mann–Whitney U test followed by a Bonferroni correction, Figure 6). On day 14, the proportion was 13.1% in the Sham-operated group, 22.1% in the NGF50 group, and 34.7% in the NGF100 group. The proportion increased dose dependently (Sham versus NGF50, \( P = 0.0001 \); NGF50 versus NGF100, \( P = 0.0005 \); NGF100 versus Sham, \( P = 0.0001 \); respectively, Mann–Whitney U test followed by a Bonferroni correction, Figure 6). By contrast, the proportions in each group were not significantly different between days 7 and 14.

Discussion

We demonstrated that intra-articular injection of NGF into the rat hip produced microinflammation without osteoarthritic change. To our knowledge, this is the first report describing an NGF-induced rat model of hip pain. Ashrafts et al. [17] reported intra-articular injection of NGF into the rat OA knee. OA was induced in rat knees by meniscal transection or intra-articular monosodium iodoacetate injection, and then NGF added to evoke pain-related behavior. However, there are no reports to our knowledge of intra-articular injection of NGF into rat normal hips. We found invasion of inflammatory cells in synovial tissue and angiogenesis in rat hips. Walsh et al. [18] also described angiogenesis and NGF at the
osteoarthritis. By contrast, we did not observe any joint space narrowing or cartilage degeneration. Furthermore, invasion of inflammatory cells in synovial tissue and angiogenesis were sustained for at least until 14 days after NGF application. NGF is a neurotrophin that regulates neuronal development, survival, and maintenance [19]. NGF plays a key role in many persistent pain states, notably those associated with inflammation [20–23]. We hypothesize that direct application of NGF into the hip evokes an inflammatory condition without degeneration of the articular cartilage and that NGF can be used to model inflammatory hip pain in rats.

In the current study, CGRP was upregulated in DRG neurons by intra-articular injection of NGF. CGRP is a marker of sensory neurons typically involved in nociception [24–25]. Pain from the hip is most likely transmitted by CGRP-IR DRG neurons [9]. Orita et al. reported that the concentration of NGF was significantly elevated after 7 days in synovium in a rat model of knee MIA [26]. NGF is retrogradely transported to DRG and mediates CGRP production. Thus, intra-articular injection of NGF influenced nociceptive transmission at the DRG via production of CGRP. However, it is interesting that the NGF50 group showed a significantly lower proportion of CGRP-IR neurons than the NGF100 group despite a similar extent of inflammation. Further study is needed to determine the minimum amount of NGF that causes inflammatory change and increase in CGRP.

The mechanism of pain transmission in hip disorders is not completely understood. Nakajima et al. [9] reported that CGRP-IR neurons play an important role in the perception of pain in the hip joint in rats. They also documented that FG-labeled neurons were distributed throughout the ipsilateral DRGs from T13 to L5, primarily at L1, L2, L3, and L4 in the hip joint. The current study showed almost similar distributions and the distribution was not changed despite NGF administration. These results explain the clinical observation that pain is referred to originate in the thigh and lower leg in patients with disorders of the hip [6]. In patients with OA of the hip, sensory innervation and inflammatory cytokines in hypertrophic synovia are associated with nociception [14,27]. Shirai et al. [28] stated that hip pain occurs following the invasion of the labrum with blood vessels and nerve fibers from inflamed synovial tissue following labral degeneration in the hip joint affected by OA. Shigemura et al. [2] reported that 6% of patients with OA of the hip showed neuropathic pain. These findings may provide the development of novel analgesic therapies for hip joint pain including the treatments targeting NGF. Recently, a randomized clinical trial of an NGF inhibitor in OA of the hip and knee was reported to reduce joint pain [29–31].

There are several limitations to this study. First, the distribution of cytokines was not examined in histopathology of the hip. The hip joint is a complex structure consisting of synovium, cartilage, and bone. The level of inflammatory cytokines and their receptors, the macrophages and endothelial cell markers in each structure should be investigated quantitatively using protein determination or gene expression. Second, hip pain was suggested by immunohistochemical determination of CGRP expression. Further study is needed to determine pain-related behavior using other methods such as evaluation of weight bearing in walking animals [32–33]. Third, dose dependency could not be found, since only NGF50 and NGF100 groups were documented. Higher dose and lower dose NGF models should be studied in the future.

In conclusion, a novel rat model of hip pain was produced by intra-articular administration of NGF into the hip joint. NGF elicited mild synovitis and expression of CGRP-IR in sensory nerves. We suggest that NGF is crucially involved in the expression and transmission of pain from the hip joint.

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Conflict of interest
None.

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