Eur Spine J (2015) 24:2017–2022
DOI 10.1007/s00586-015-4058-z

ORIGINAL ARTICLE

Anti-RANKL antibodies decrease CGRP expression in dorsal root ganglion neurons innervating injured lumbar intervertebral discs in rats

Masashi Sato[1] · Kazuhide Inage[1] · Yoshihiro Sakuma[1] · Jun Sato[1] · Sumihisa Orita[1] · Kazuyo Yamauchi[1] · Yawara Eguchi[1] · Nobuyasu Ochiai[1] · Kazuki Kuniyoshi[1] · Yasuchika Aoki[1] · Junichi Nakamura[1] · Masayuki Miyagi[1] · Miyako Suzuki[1] · Gou Kubota[1] · Takeshi Sainoh[1] · Kazuki Fujimoto[1] · Yasuhiro Shiga[1] · Koki Abe[1] · Hiroto Kanamoto[1] · Gen Inoue[1] · Kazuhisa Takahashi[1] · Seiji Ohtori[1]

Received: 31 December 2014 / Revised: 26 May 2015 / Accepted: 7 June 2015 / Published online: 14 June 2015

Abstract

Purpose Nuclear factor-κB (NF-κB), receptor activator of NF-κB (RANK), and RANK ligand (RANKL) are transcriptional regulators of inflammatory cytokines. RANKL expression in dorsal root ganglion (DRG) neurons is elevated in animal models of pain or intervertebral disc herniation. We sought to evaluate the effect of anti-RANKL antibodies on sensory nerves innervating injured intervertebral discs.

Method We labeled DRG neurons innervating L5-6 discs with FluoroGold (FG). The L5-6 discs of 36 rats were punctured using a 23-gage needle and 18 rats underwent sham surgery without disc puncture. The puncture group was evenly subdivided into a group in which 10 μl saline was administered to the injured disc and a group in which 10 μl of anti-RANKL antibody was administered. Seven and 14 days postsurgery, DRGs at L2 level were harvested, sectioned, and immunostained for calcitonin gene-related peptide (CGRP). The proportion of CGRP-immunoreactive (IR) DRG neurons of all FG-positive neurons was determined. Amount of tumor necrosis factor (TNF)-α and interleukin(IL)-6 was measured within the intervertebral discs in each group at 7 and 14 days after surgery using an enzyme-linked immunosorbent assay (ELISA).

Results The proportion of CGRP-IR DRG neurons to total FG-labeled neurons innervating injured intervertebral discs and amount of TNF-α and IL-6 in the injured discs in the saline control group was significantly increased compared with that found in rats from the sham surgery group ($P < 0.05$). However, application of anti-RANKL antibody to the injured discs significantly decreased the proportion of CGRP-IR DRG neurons to total FG-labeled neurons and amount of TNF-α and IL-6 in the injured discs ($P < 0.05$).

Conclusions TNF-α and IL-6 in the injured discs increased and CGRP expression increased in DRG neurons innervating injured discs, and antibodies to RANKL could suppress this increased TNF-α, IL-6, and CGRP expression. RANKL may be a therapeutic target for pain control in patients with lumbar disc degeneration.

Keywords Pain · Nerve · Intervertebral disc · RANKL · CGRP

Introduction

Low back pain is a common clinical problem and a significant socioeconomic problem. At any one time, from 15 to 30 % of the population has low back pain; the 1-month incidence is from 19 to 43 % of the population; and the lifetime incidence is up to 80 % of the population [1]. Intervertebral discs are thought to be a source of non-specific low back pain [1]. However, the pathophysiology of discogenic low back pain is not well understood. Low back pain is characterized by a confluence of innervation, inflammation, and mechanical hypermobility [2, 3]. Elevated levels of various proinflammatory molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, inducible nitric oxide synthase, prostaglandin E_2, and nerve growth factor have been found in intervertebral discs in various animal models of discogenic pain and degenerated intervertebral discs from humans [2, 3].

✉ Seiji Ohtori
sohtori@faculty.chiba-u.jp

[1] Department of Orthopaedic Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Transcription factor nuclear factor (NF)-κB plays a crucial role in regulating proinflammatory cytokine gene expression. In animal models, NF-κB is activated in dorsal root ganglia (DRG) after partial sciatic nerve injury or disc injury and plays a crucial role in hyperalgesia [4–6]. In these conditions mimicking inflammatory disease, there is increased production of proinflammatory cytokines such as TNF-α and IL-6, NF-κB, receptor activator of NFκB (RANK), and its ligand (RANKL) [7–9].

RANKL expression in DRG neurons is elevated in a rat model of intervertebral disc herniation [10].

Animal and human studies show sensory nerve fibers contain substance P (SP) and calcitonin gene-related peptide (CGRP) [11, 12]. Changes in SP and CGRP levels in DRGs innervating animal intervertebral discs are often investigated as putative markers related to inflammatory pain [13, 14]. In the present study, we sought to determine the effect of anti-RANKL antibodies on CGRP expression in sensory nerves innervating injured intervertebral discs in rats.

Materials and methods

All protocols for animal procedures were approved by the Ethics Committee of Chiba University.

Retrograde labeling of DRG neurons innervating L5-6 discs with FluoroGold

Thirty-six male Sprague–Dawley (SD) rats weighing 220–250 g were used (8-week old at the start of experiments; Japan SLC, Shizuoka, Japan). All animals were housed in the animal unit at 21 °C on a 12-h light–dark cycle (08:30–20:30 h) and fed a pellet diet and tap water ad libitum before and after surgery. After anesthesia with sodium pentobarbital, four crystals of FluoroGold neurotracer (FG; Fluorochrome, Denver, CO) were applied to the surfaces of the L5-6 intervertebral discs to label the DRG neurons innervating the discs. Immediately after FG application, the intervertebral discs in 24 rats were punctured five times with a 23-gage needle (puncture group). The 12 remaining rats were used as sham surgery controls (sham surgery group). The 24 rats in the puncture group were evenly subdivided into a group in which 10 μl of saline was administered to the disc (puncture + saline group) ($n = 12$) and a group in which 10 μl of anti-RANKL antibody (200 μg/ml) was administered (Santa Cruz Biotechnology, Dallas, TX) (puncture + anti-RANKL group) 3 min after puncture during the same surgery. The epitope mapping is at the N-terminus of RANKL. The hole was immediately sealed with cyanoacrylate adhesive to prevent leakage of anti-RANKL antibody or saline, and the skin was closed. This procedure was performed following previously reported methods [13, 14].

Calcitonin gene-related peptide (CGRP) immunohistochemistry in DRGs

CGRP immunoreactivity of DRG neurons was determined at 7 and 14 days after injury ($n = 6$ each for each group at each time). After anesthesia with sodium pentobarbital, rats were transcardially perfused with 500 ml of 4 % paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Using a dorsal approach, back muscle and lamina were removed, and bilateral L2 DRGs were resected (sensory neurons in these DRGs innervate the L5-6 disc [13, 14]). After soaking in 0.01 M phosphate buffered saline (PBS) containing 20 % sucrose for 20 h at 4 °C to cryoprotect the tissue, each ganglion was sectioned at 10-μm thickness on a cryostat and resultant sections mounted on poly-L-lysine-coated slides.

Subsequently, the sections were incubated with rabbit anti-CGRP (1:1000; Chemicon, Temecula, CA), diluted 1:1000 in blocking solution, for 20 h at 4 °C, followed by incubation with goat anti-rabbit Alexa Fluor 488 fluorescent antibody conjugate (1:400; Molecular Probes, Eugene, OR) to detect CGRP immunoreactivity. Ten sections were selected at random for evaluation. The sections were examined using a fluorescence microscope (Nikon, Japan). The number of FG-labeled neurons, and FG-labeled and CGRP-immunoreactive (IR) neurons was counted. The number of neurons per 0.45 mm^2 was counted at 400× magnification using a counting grid.

Measurement of inflammatory response (enzyme-linked immunosorbent assay)

Twelve SD rats weighing 220–250 g were used (8-week old at the start of experiments; Japan SLC, Shizuoka, Japan). Inflammatory cytokines were measured at days 7 and 14 after the first surgery. For each group, each three animals were sacrificed at days 7 and 14 after the first surgery (sham surgery group, L5-6 disc puncture + saline group, and L5-6 disc puncture + anti-RANKL group). After anesthesia with sodium pentobarbital, intervertebral L5-6 discs were harvested for evaluation with enzyme-linked immunosorbent assay (ELISA). Tumor necrosis factor (TNF)-α and interleukin (IL)-6 production in the intervertebral discs were quantified using an ELISA in accordance with the manufacturer's protocol (R&D Systems, Minneapolis, MN). Tissue protein was assayed using a kit in accordance with the manufacturer's protocols (Bio-Rad, Hercules, CA), and inflammatory mediator levels were normalized to tissue protein levels.

Statistical analysis

Amount of TNF-α and IL-6 production, and the average proportions of FG-labeled and FG-labeled CGRP-positive neurons were compared between groups using Welch's unpaired t test. $P < 0.05$ was considered statistically significant. Results are reported as mean ± SEM.

Results

FG-labeled DRG neurons innervating L5-6 discs

FG-labeled DRG neurons innervating L5-6 discs were present in bilateral L2 DRGs (Table 1; Fig. 1). There were no significant differences between the three groups in the distribution of FG-labeled neurons between the right and left side, at either 7 or 14 days (Table 1).

Ratio of FG-labeled CGRP-IR DRG neurons to FG-labeled neurons innervating L5-6 discs

Figure 1 shows FG-labeled and FG-labeled CGRP-IR neurons at L2 in the three groups on day 7. Figure 2 shows the ratio of FG-labeled CGRP-IR neurons to FG-labeled neurons at L2 in the three groups at 7 and 14 days. The ratio of CGRP-IR DRG neurons to total FG-labeled neurons at L2 in the puncture + saline group significantly increased at 7 and 14 days, respectively (55 ± 10 and 62 ± 12 %, mean ± S. E.) compared with the sham surgery or puncture + anti-RANKL group at 7 and 14 days after surgery, respectively (sham surgery, 30.4 ± 4 and 33.4 ± 9 %, $P < 0.05$; puncture + anti-RANKL, 40 ± 7 and 45.8 ± 7 %, $P < 0.05$). The ratio of CGRP-IR DRG neurons to total FG-labeled neurons in the puncture + anti-RANKL group was significantly greater at 7 and 14 days compared with the sham surgery group ($P < 0.05$).

Table 1 Number of FG-labeled neurons at L2

	L2 (right)	L2 (left)	*P*
7 day			
Sham surgery	165	148	N.S.
Puncture + saline	167	152	N.S.
Puncture + anti-RANKL	170	160	N.S.
14 day			
Sham surgery	150	133	N.S.
Puncture + saline	140	132	N.S.
Puncture + anti-RANKL	126	111	N.S.

N.S. not significant

The behavior of inflammatory mediators

Figure 3 shows TNF-α and IL-6 levels in the three groups on day 7 and 14. Both TNF-α and IL-6 levels on day 7 and 14 were significantly higher in the puncture + saline group compared with sham surgery group ($P < 0.05$). However, both TNF-α and IL-6 levels on day 7 and 14 were significantly lower in the puncture + anti-RANKL group compared with the puncture + saline group ($P < 0.05$).

Discussion

In the present study, we showed that CGRP-IR in disc-innervating DRG neurons at L2 increased after disc injury. However, antibodies to RANKL suppressed CGRP-IR in these DRG neurons. Furthermore, antibodies to RANKL suppressed both TNF-α and IL-6 levels in injured intervertebral discs.

Many studies have investigated lumbar intervertebral discs as a possible source of low back pain. The dorsal portion of the rat L5-6 disc is multisegmentally innervated by T13 to L6 DRG neurons [15, 16]. Of the sensory DRG neurons innervating the L5-6 disc, most derive from the L2 DRG. About half of the DRG neurons innervating the rat L5-6 disc or human L4-5 disc are immunoreactive for SP or CGRP, which are well known as neurotransmitters involved in inflammatory pain [17–19]. Blockade of the spinal nerves at the same level is effective for some patients with lumbar discogenic pain, but for other patients, blockade of L2 spinal nerves is effective [20, 21]. In the present study, we examined only L2 DRG because the rat L5-6 disc is innervated mainly by L2 DRG. Indeed, FG retrograde labeling in the present study showed that many neurons in L2 DRG innervated the L5-6 disc.

In the current study, TNF-α and IL-6 levels increased in injured intervertebral discs and the expression of CGRP increased in DRG neurons innervating injured discs, and antibodies to RANKL could suppress this increase of cytokines and CGRP expression. We speculate that NF-κB regulates the expression of genes for proinflammatory cytokines, such as TNF-α and IL-6, NF-κB, RANK, and RANKL in injured site and in the nerves [7–9]. Indeed, at least some of these cytokines, including NF-κB and RANKL, are activated in DRG in models of partial sciatic nerve injury or disc herniation [4–6, 10]. TNF-α dose dependently induces SP, an inflammatory neuropeptide, in the nervous system [22]. TNF-α induces the release of inflammatory neuropeptides, SP, and CGRP from peripheral terminals and enhances heat-evoked release of CGRP from peripheral nerves [22–24]. Application of disc tissue including TNF-α onto nerve roots induces an increase of

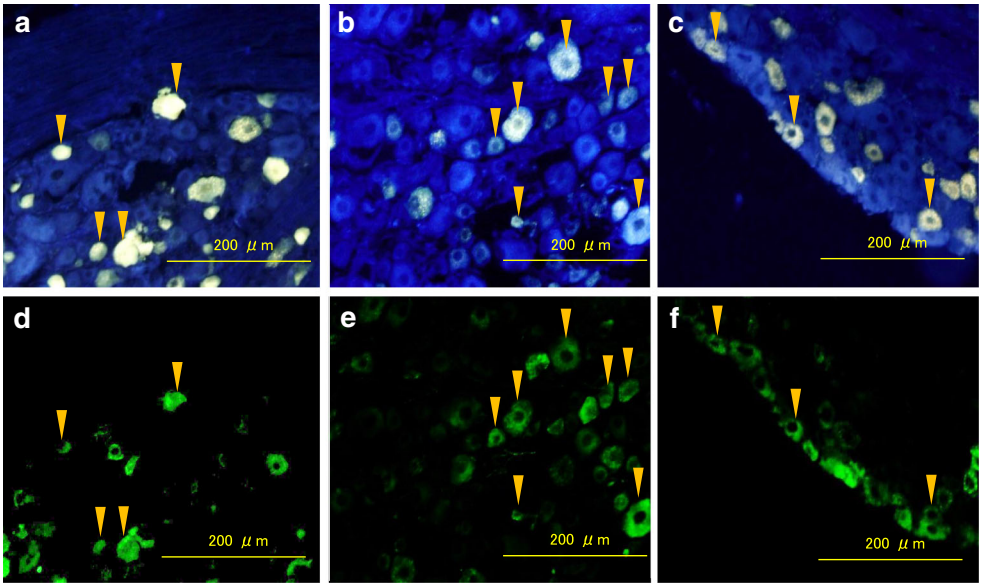

Fig. 1 FluoroGold (FG)-labeled dorsal root ganglion (DRG) neurons in the sham surgery group (**a**), the puncture + saline group (**b**), and the puncture + receptor activator of nuclear factor-κB ligand (RANKL) antibody group (**c**). Calcitonin gene-related peptide (CGRP)-immunoreactive (IR) DRG neurons in each group (**d**, **e**, and **f**). **a** and **d**, **b** and **e**, and **c** and **f** are the same sections, respectively. *Arrowheads* indicate DRG neurons that are both FG-labeled and CGRP-IR

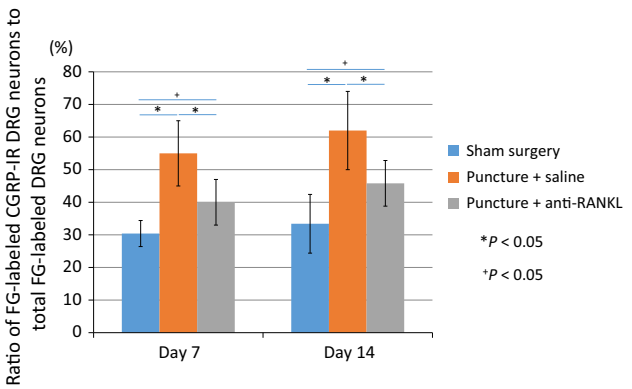

Fig. 2 Fractional ratio of FluoroGold (FG)-labeled calcitonin gene-related peptide (CGRP)-immunoreactive (IR) L2 dorsal root ganglion (DRG) neurons to FG-labeled DRG neurons in non-puncture, puncture + saline, and puncture + receptor activator of nuclear factor-κB ligand (RANKL) antibody groups on day 7 and 14. The ratio in the puncture + saline group was significantly greater than in the sham surgery and the puncture + anti-RANKL groups on both days 7 and 14 ($^{*}P < 0.05$). The ratio in the puncture + anti-RANKL group was significantly greater than in the sham surgery group on both days 7 and 14 ($^{+}P < 0.05$)

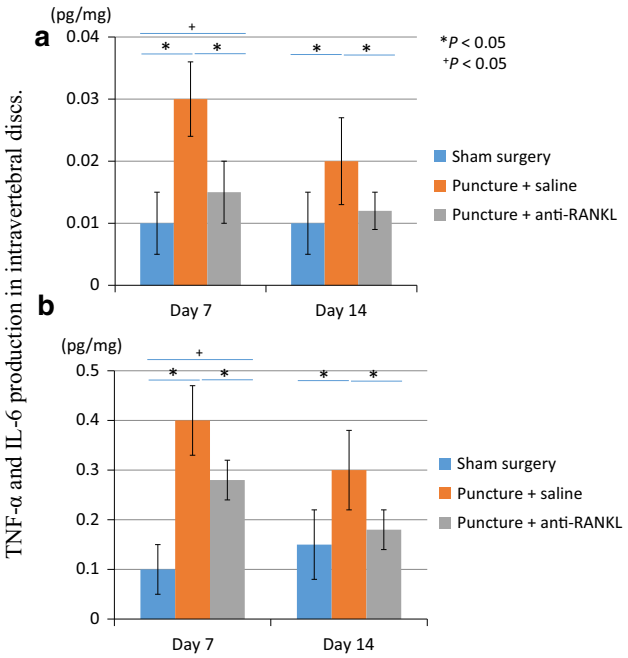

Fig. 3 TNF-α (**a**) and IL-6 (**b**) production in intravertebral discs in the three groups on day 7 and 14. Both TNF-α and IL-6 levels on day 7 and 14 were significantly higher in the puncture + saline group compared with sham surgery and the puncture + anti-RANKL groups ($P < 0.05$). Both TNF-α and IL-6 levels on day 7 were significantly higher in the puncture + anti-RANKL group compared with sham surgery group ($P < 0.05$)

brain-derived neurotrophic factor (BDNF), which is colocalized with CGRP in DRG neurons, but TNF-α inhibitor reduced the BDNF production in DRGs in this model [25]. NF-kB decoy oligodeoxynucleotides are effective in suppressing inflammatory cytokine expression [26, 27]. In several inflammatory diseases, such as menopausal osteoporosis, rheumatoid arthritis, and periodontitis, there is increased production of the proinflammatory cytokines TNF-α, NF-kB, and RANKL [8, 28, 29]. Induction of neuropeptides associated with inflammation, such as SP and CGRP, is regulated by inflammatory cytokines. These

cytokines are regulated by proinflammatory cytokines NF-kB and RANK. The inhibitory effect of CGRP on DRG neurons by antibodies to RANKL may be explained by these effects.

IL-6, TNF-α, NF-κB, RANK, and RANKL are important mediators of pain and may be therapeutic targets for pain control in animal models and humans. A prospective randomized study showed that epidural administration of spinal nerves with the TNF-α inhibitor, etanercept, or anti-IL-6 receptor antibody, tocilizumab, was more effective for treatment of sciatica in patients with lumbar spinal stenosis than dexamethasone [30, 31]. NF-κB decoy (inhibitor) could be introduced into DRG neurons effectively in in vitro and in vivo models, and suppressed pain-related behavior in a rat model of inflammatory foot pain [5]. Furthermore, NF-κB decoy suppressed markers of inflammatory and neuropathic pain in a rat model of lumbar disc herniation [6]. Inhibition of RANKL is effective for pain relief in bone disease [32–34]. Inhibitors of RANKL–RANK interaction (e.g., denosumab) have been used for treatment of painful osseous metastases [32]. Denosumab was found to decrease the size of giant cell tumors of the bone, and reduce pain from these tumors in an open label, phase 2 study [32]. In a large study of postmenopausal women with osteoporosis, denosumab reduced pain related to osteoporosis and the risk of vertebral, nonvertebral, and hip fractures compared with placebo over 3 years [34]. Cytokines are important inducers of pain, including that originating from osteoporotic bone. Bisphosphonates, which are used to treat osteoporosis, affect pain from osteoporosis, because they inhibit osteoclast activity and have an antiinflammatory effect [35, 36]. In vitro studies have shown that bisphosphonates inhibit the synthesis of proinflammatory cytokines including TNF-α and interleukins [37]. Blockade of RANKL may therefore be an effective therapeutic strategy for the treatment of pain originating from intervertebral discs.

Our study has several limitations. First, we did not examine the expression of RANKL, or other neuropeptides and cytokines, in the injured discs or DRG neurons innervating the discs. Second, we did not evaluate pain directly, for example using pain-related behavioral studies, because this is notoriously difficult in animal models of low back pain. Further studies are needed to examine the role of RANKL in discogenic low back pain more directly.

In conclusion, cytokines induction increased in injured discs and CGRP expression increased in DRG neurons innervating injured discs, and antibodies to RANKL could suppress this increase in the cytokines and CGRP expression. RANKL may be a therapeutic target for pain control in patients with lumbar disc degeneration.

Conflict of interest The authors declare that there is no conflict of interest for this research.

References

1. Nachemson A (2004) Epidemiology and the economics of low back pain. In: Herkowits HN, Dvorak J, Bell G, Nordin M, Grob D (eds) The Lumbar Spine, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, pp 3–10
2. Lotz JC, Ulrich JA (2006) Innervation, inflammation, and hypermobility may characterize pathologic disc degeneration: review of animal model data. J Bone Joint Surg Am 88:76–82
3. Ohtori S, Inoue G, Miyagi M et al. (2014) Pathomechanisms of discogenic low back pain in humans and animal models. Spine J. pii:S1529–9430(14)00279–4
4. Ma W, Bisby MA (1998) Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. Brain Res 797:243–254
5. Inoue G, Ochiai N, Ohtori S et al (2006) Injection of nuclear factor-kappa B decoy into the sciatic nerve suppresses mechanical allodynia and thermal hyperalgesia in a rat inflammatory pain model. Spine 31:2904–2908
6. Suzuki M, Inoue G, Gemba T et al (2009) Nuclear factor-kappa B decoy suppresses nerve injury and improves mechanical allodynia and thermal hyperalgesia in a rat lumbar disc herniation model. Eur Spine J 18:1001–1007
7. Charatcharoenwitthaya N, Khosla S, Atkinson EJ et al (2007) Effect of blockade of TNF-α and interleukin-1 action on bone resorption in early postmenopausal women. J Bone Miner Res 22:724–729
8. Eghbali-Fatourechi G, Khosla S, Sanyal A et al (2003) Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. J Clin Invest 111:1221–1230
9. Feng X (2005) Regulatory roles and molecular signaling of TNF family members in osteoclasts. Gene 350:1–13
10. Matsuyama Y, Sakuma Y, Suzuki M et al (2014) Evaluation of behavior and expression of receptor activator of nuclear factor-kappa B ligand in dorsal root ganglia after sciatic nerve compression and application of nucleus pulposus in rats. Asian Spine J 8:557–564
11. Ashton IK, Walsh DA, Polak JM et al (1994) Substance P in intervertebral discs: binding sites on vascular endothelium of the human annulus fibrosus. Acta Orthop Scand 65:635–639
12. McCarthy PW, Carruthers B, Martin D et al (1991) Immunohistochemical demonstration of sensory nerve fibres and endings in the lumbar intervertebral discs of the rat. Spine 16:653–655
13. Miyagi M, Millecamps M, Danco AT et al (2014) ISSLS Prize winner: increased innervation and sensory nervous system plasticity in a mouse model of low back pain due to intervertebral disc degeneration. Spine 39:1345–1354
14. Horii M, Orita S, Nagata M et al (2011) Direct application of the tumor necrosis factor-α inhibitor, etanercept, into a punctured intervertebral disc decreases calcitonin gene-related peptide expression in rat dorsal root ganglion neurons. Spine. 36:E80–E85
15. Ohtori S, Takahashi K, Chiba T et al (2001) Sensory innervation of the dorsal portion of the lumbar intervertebral discs in rats. Spine 26:946–950
16. Ohtori S, Takahashi Y, Takahashi K et al (1999) Sensory innervation of the dorsal portion of the lumbar intervertebral disc in rats. Spine 24:2295–2299
17. Ohtori S, Takahashi K, Chiba T et al (2002) Substance P and calcitonin gene-related peptide immunoreactive sensory DRG

neurons innervating the lumbar intervertebral discs in rats. Ann Anat 183:235–240
18. Ozawa T, Aoki Y, Ohtori S et al (2003) The dorsal portion of the lumbar intervertebral disc is innervated primarily by small peptide-containing dorsal root ganglion neurons in rats. Neurosci Lett 344:65–67
19. Ozawa T, Ohtori S, Inoue G et al (2006) The degenerated lumbar intervertebral disc is innervated primarily by peptide-containing sensory nerve fibers in humans. Spine 31:2418–2422
20. Nakamura S, Takahashi K, Takahashi Y et al (1996) Origin of nerves supplying the posterior portion of lumbar intervertebral discs in rats. Spine 21:917–924
21. Ohtori S, Nakamura S, Koshi T et al (2010) Effectiveness of L2 spinal nerve infiltration for selective discogenic low back pain patients. J Orthop Sci 15:731–736
22. Ding M, Hart RP, Jonakait GM (1995) Tumor necrosis factor-α induces substance P in sympathetic ganglia through sequential induction of interleukin-1 and leukemia inhibitory factor. J Neurobiol 28:445–454
23. Hua XY, Chen P, Fox A et al (1996) Involvement of cytokines in lipopolysaccharide-induced facilitation of CGRP release from capsaicin-sensitive nerves in the trachea: studies with interleukin-1β and tumor necrosis factor-α. J Neurosci 16:4742–4748
24. Oprée A, Kress M (2000) Involvement of the proinflammatory cytokines tumor necrosis factor-α, IL-1β, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. J Neurosci 20:6289–6293
25. Onda A, Murata Y, Rydevik B et al (2004) Infliximab attenuates immunoreactivity of brain-derived neurotrophic factor in a rat model of herniated nucleus pulposus. Spine 29:1857–1861
26. Morishita R, Sugimoto T, Aoki M et al (1997) In vivo transfection of cis element ''decoy'' against nuclear factor-κB binding site prevents myocardial infarction. Nat Med 8:894–899
27. Sakurai H, Shigemori N, Hisada Y et al (1997) Suppression of NF-κB and AP-1 activation by glucocorticoids in experimental glomerulonephritis in rats: molecular mechanisms of anti-nephritic action. Biochim Biophys Acta 1362:252–262
28. Novack DV, Teitelbaum SL (2008) The osteoclast: friend or foe? Ann Rev Pathol 3:457–484
29. Boyce BF, Xing L (2008) Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys 473:139–146
30. Ohtori S, Miyagi M, Eguchi Y et al (2012) Epidural administration of spinal nerves with the tumor necrosis factor-alpha inhibitor, etanercept, compared with dexamethasone for treatment of sciatica in patients with lumbar spinal stenosis: a prospective randomized study. Spine 37:439–444
31. Ohtori S, Miyagi M, Eguchi Y et al (2012) Efficacy of epidural administration of anti-interleukin-6 receptor antibody onto spinal nerve for treatment of sciatica. Eur Spine J 21:2079–2084
32. Smith HS, Barkin RL (2014) Painful Boney Metastases. Am J Ther 21:106–130
33. Thomas D, Henshaw R, Skubitz K et al (2010) Denosumab in patients with giant-cell tumour of bone: an open-label, phase 2 study. Lancet Oncol 11:275–280
34. Moen MD, Keam SJ (2011) Denosumab: a review of its use in the treatment of postmenopausal osteoporosis. Drugs Aging 28:63–82
35. Bonabello A, Galmozzi MR, Bruzzese T et al (2001) Analgesic effect of bisphosphonates in mice. Pain 91:269–275
36. Fulfaro F, Casuccio A, Ticozzi C et al (1998) The role of bisphosphonates in the treatment of painful metastatic bone disease: a review of phase III trials. Pain 78:157–169
37. Van Offel JF, Schuerwegh AJ, Bridts CH et al (2001) Influence of cyclic intravenous pamidronate on proinflammatory monocytic cytokine profiles and bone density in rheumatoid arthritis treated with low dose prednisolone and methotrexate. Clin Exp Rheumatol 19:13–20