**RANK/RANKL/OPG System in the Rat Intervertebral Disc**

Norihiko Takegami, MD, Koji Akeda, MD, PhD, Koichiro Murata, MD, Junichi Yamada, MD, Akihiro Sudo, MD, PhD.

Mie University Graduate School of Medicine, Tsu, Japan.

**Disclosures:** N. Takegami: None. K. Akeda: None. K. Murata: None. J. Yamada: None. A. Sudo: None.

**Introduction:** The homeostatic imbalance of matrix metabolism induced by the local production of proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and matrix-degrading enzymes, is thought to contribute to the biochemical changes in intervertebral disc (IVD) degeneration [1]. The receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), a member of the TNF ligand superfamily, is well known to regulate bone metabolism. RANKL and its receptor (RANK) have been shown to play a crucial role in osteoclast differentiation and activation. Binding of RANKL to RANK activates TNF receptor-associated factor (TRAF) 6, which stimulates the expression of proinflammatory cytokines through NF-κB pathways [2]. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL preventing it from binding to RANK. It has recently been reported that RANKL is also expressed by human articular cartilage and IVD [3, 4, 5]. However, the expression of each component of the RANK/RANKL/OPG system in the IVD has not been examined in detail. Therefore, the purpose of this study was (1) to examine the mRNA and immunohistochemical expression of the RANK/RANKL/OPG system in the IVD, (2) to examine the expression of RANK/RANKL/OPG system under the stimulation of IL-1β, and (3) to evaluate the effect of RANKL on the expression of proinflammatory cytokine with/without IL-1β stimulation.

**Methods:** 12-week-old male Sprague-Dawley rats (n=90) were used in this study.

**Cell Isolation:** The annulus fibrosus (AF) and the nucleus pulposus (NP) were carefully dissected from thoracolumbar discs and cells were isolated by sequential enzyme digestion. The cells were washed with Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DMEM/F12) and the primary culture was started. Isolated cells were cultured in monolayer at 4.0×10⁴ cells/mL with 5% CO₂, 95% air in complete medium (DMEM/F12 containing 10% fetal bovine serum (FBS), 25μg/mL ascorbic acid, and 50μg/mL gentamicin).

**Immunohistochemistry of IVD cells:** After preculture on 4-chamber slides for 7 days (AF) or 14 days (NP), the cells were incubated with anti-RANK, anti-RANKL or anti-OPG antibodies. Secondary Alexa 488-conjugated anti-mouse immunoglobulin G (IgG), anti-rabbit IgG or anti-goat IgG antibodies were applied. The nuclei were stained with propidium iodide and cover-slipped with Vectashield mounting medium. Samples were imaged using confocal microscopy.

**The effect of IL-1β stimulation on the mRNA levels of RANK/RANKL/OPG:** After preculture, the AF or NP cells were cultured in serum-free medium for 24 hours. The cells were then cultured with or without recombinant human IL-1β (rhIL-1β) at 0.01, 0.1, 1.0 or 10 ng/ml in DMEM/F12 containing 0.3% FBS for an additional 24 hours.

**The effect of RANKL on the mRNA expression of proinflammatory cytokine with or without IL-1β:** After preculture, the AF or NP cells were cultured in serum-free medium for 24 hours. The cells were then cultured with or without rhRANKL at 10 or 100 ng/ml in the presence or absence of rhIL-1β (1.0ng/ml) in DMEM/F12 containing 0.3% FBS for an additional 24 hours.
RNA isolation: Total RNA was isolated from rat AF and NP cells in monolayer culture using Isogen (Nippon Gene, Toyama, Japan), according to the manufacturer’s instructions. Total RNA was reverse-transcribed using the first-strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) with the DNA thermal cycler (Veriti; Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. The assay was calibrated using 18S ribosomal RNA as an internal control.

Results: Immunohistochemical analysis of rat IVD cells: In monolayer cultures of rat AF and NP cells, immunoreactivity to RANK, RANKL and OPG was clearly identified. Confocal images revealed that immunoreactivity to RANK was mainly found in cell membranes of both AF and NP cells. The expression of RANKL was distributed in the cytoplasm of both AF and NP cells. OPG immunoreactivity was found in a spot-like distribution in the cytoplasm of AF and NP cells. No immunoreactivity was found in the isotype controls.

mRNA expression of RANK/RANKL/OPG by rat IVD cells: mRNA expression levels of RANK, RANKL and OPG were clearly identified by both rat AF and NP cells. There were no significant differences on those expression levels between AF and NP cells.

The effect of IL-1β on mRNA levels of RANK/RANKL/OPG: The mRNA expression level of RANKL was significantly up-regulated by stimulation with IL-1β in both AF and NP cells (Figure). The mRNA expression level of RANK in NP cells was up-regulated by stimulation with IL-1β. However, no significant changes were identified in AF cells. There was no significant change in the mRNA expression of OPG by stimulation with IL-1β in both AF and NP cells.

The effect of RANKL on the mRNA expression of proinflammatory cytokines with or without IL-1β: Treatment with RANKL did not induce a significant effect on the mRNA expression of IL-1β by AF cells. However, in AF cells the mRNA expression of IL-1β was significantly up-regulated by the stimulation of RANKL with IL-1β (1.0ng/ml). Similar results were obtained for NP cells.

Discussion: The present study showed that the expression of the RANK/RANKL/OPG system was confirmed both by mRNA and protein levels in the rat IVD. The expression of RANKL was regulated by stimulation with IL-1β both by rat AF and NP cells. Interestingly, the mRNA expression level of IL-1β was significantly up-regulated by stimulation with RANKL in the presence of IL-1β stimulation. The results of this study suggest that RANKL has the potential to accelerate the expression of proinflammatory cytokines in the presence of IL-1β. The RANK/RANKL/OPG system may play a role in the process of disc degeneration mediated by proinflammatory cytokines.

Significance: The mRNA expression level of IL-1β was significantly up-regulated by stimulation with RANKL in the presence of proinflammatory cytokines. The RANK/RANKL/OPG system and proinflammatory stimuli may play a role in the progression of disc degeneration.