Age-related of MCP-1 and MMP-3 expression in intervertebral disc in relation to inflammatory cytokine stimulation

Fujita, K; 1Ando, T; 1Obha, T; 1Wako, M; 2Kato, R; 1Nakao, A; +1Haro, H
+1Dept of Orthopaedic surgery, 1Dept of Human Pathology, 2Dept of Immunology, University of Yamanashi, JAPAN, haro@yamanashi.ac.jp

New Significance
Aging is associated with a reduction of positive-cell numbers and expressions of MCP-1 and MMP-3 in intervertebral disc (IVD). The response to IVD inflammation also decreased as age increased. In addition, MMP-3 plays a key role in IVD degeneration.

Introduction
HD is a common problem that is responsible for symptoms in up to 40% of all patients with low back pain. The natural resorption of HD in patients is associated with a prominent infiltration of macrophages. TNF-α produced by macrophages induces HD tissues to produce MMP-3, MCP-1 and VEGF. MMP-3 leads to significant mass reduction, extensive depletion of proteoglycan and resorption of HD tissues. VEGF also leads to neo-vascularization of HD tissues via the NF-kB pathway. MCP-1 plays a critical role in HD resorption by triggering macrophage infiltration into HD tissue via the PI3K pathway. The purpose of the current study was to investigate the sequential changes of MCP-1 and MMP-3 after treatment with the inflammatory cytokines TNF-α or TWEAK by molecular biological analysis and histological analysis of young (4-8 week-old), adult (16 week-old), and old (32-64 week-old) mice. We also examined the specific role of MMP-3 in disc degeneration with MMP-3-deficient mice.

Methods
Mice
Homozygous wild type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MMP-3-deficient mice were generated by homologous recombination as described previously and maintained on a 129/SvEv background. Experimental protocols were approved by the Institutional Animal Care and Use Committee of our University (No 19-105).

Disc organ culture
Coccygeal intervertebral disc tissue specimens were obtained from the tail bone using a dissecting microscope after the skin and soft tissue were removed. For organ culture assays, microscopy was used to harvest intervertebral disc tissue completely from the end plates. Whole intervertebral disc tissue specimens were cultured in a 24-well plate in 1 ml of DMEM containing 0.1% FBS and 50 microg/ml penicillin and streptomycin in the presence or absence of 10 ng/ml rm TNF-α or 100 ng/ml rm TWEAK for 12 or 72 h in a humidified environment of 5% CO2 at 37°C.

Quantitative real-time PCR, Western blotting, ELISA
Expression of MCP-1, VEGF, MMP-3, and GAPDH messenger RNA (mRNA) in IVD derived from 4- to 64-week-old mice was assessed by quantitative real-time PCR. The ability of MCP-1 and MMP-3 expression in IVD to respond to TNF-α or TWEAK stimulation was examined by quantitative PCR. Western blot analysis, ELISA.

Immunohistochemical staining
Sections were deparaffinized with xylene and were treated with 3% hydrogen peroxide in methanol to remove endogenous peroxidase activity, then incubated with anti-mouse MCP-1 monoclonal antibody, MMP-3 monoclonal antibody, anti-mouse aggrecan polyclonal antibody, anti-mouse type II collagen or control IgG. Immunopositive cells were evaluated by independent pathologists examined the number of cells in 5 high power fields in a blinded fashion.

Safranin-O and Alcian blue staining
Sections of IVDs derived from 4- or 64-week-old wild-type C57BL6J or MMP-3-deficient mice were stained with 0.25% Safranin-O for 5 min or Alcian blue solution for 20 min at room temperature as an indicator of proteoglycan content in tissue sections.

Statistical analysis
Data are presented as the mean ± standard deviation (SD). The significance was then determined using a Student’s or Welch’s t-test after an F-test was performed, unless otherwise stated. If the raw data did not fit a normal distribution, the Mann-Whitney U-test was used. P values less than 0.05 were considered significant.

Results
1) MCP-1, MMP-3, or VEGF mRNA expression in IVD with aging MCP-1, MMP-3 or VEGF mRNA was expressed in mouse disc tissue. Following 72h of treatment in culture, MCP-1 or MMP-3 production was significantly increased by treatment with rm TNF-α or rm TWEAK. We then elucidated age-related changes in MCP-1 or MMP-3 expression by real-time PCR of disc tissues harvested from mice of varying ages. MCP-1 or MMP-3 mRNA expression in mouse disc tissues diminished as age increased from 4 weeks to 64 weeks. Next, we elucidated whether age-related changes in MCP-1 or MMP-3 expression were induced by rm TNF-α or rm TWEAK in mouse disc tissues. MCP-1 or MMP-3 mRNA expression was assessed by real-time PCR of disc tissues from mice of varying ages cultured in the presence or absence of 10 ng/ml rm TNF-α or 100 ng/ml rm TWEAK for 72h. MCP-1 and MMP-3 mRNA expression in disc tissues diminished as age increased from 4 weeks to 64 weeks.

2) MCP-1 and MMP-3 protein expression in IVD with aging MCP-1 or MMP-3 protein expression was assessed by western blotting of cultured disc tissues and was shown to diminish as age increased from 4 and 32 weeks. MCP-1 or MMP-3 protein expression in supernatants of disc tissues gradually diminished as age increased from 4-week-old mice to 64-week-old miceMCP-1 and MMP-3 immunoreactivity in IVD by ELISA.

3) MCP-1 and MMP-3 expressions and the number of positive cells decreases with aging
Immunohistochemical analysis with anti-MCP-1 and MMP-3 antibodies revealed that these immunoreactivity was detectable in annulus fibrosus cells and nucleus pulposus cells. In addition, both positive cells were abundant in rm TNF-α or rm TWEAK-stimulated disc tissue compared with untreated samples. Both positive cells were significantly suppressed with increasing age from 4-week-old mice to 64-week-old mice in annulus fibrosus disc tissue as assessed by pathologists viewing microscope fields in an independent, blinded fashion (p<0.05).

4) Aging or MMP-3 suppression of proteoglycan synthesis
Disc tissue from 64-week-old wild mice showed very modest effects on proteoglycan loss and cartilage matrix changing compared with 4-week-old mice, whereas disc tissue from MMP-3 null mice was spared from these changing even in 64-week-old mice. Immunohistological analysis with anti-aggrecan and anti-typeII-collagen antibodies showed that these immunoreactivity are detectable in disc tissues. Although MMP-3-deficient mice at 64-week-old maintained immunoreactivity of both aggrecan and typeII collagen protein expression, wild type mice at 64-week-old reduced these expressions.

Discussion
Current our data suggest that MMP-3 is one of the target molecules involved with disc degeneration. Although we demonstrated age-related change of MCP-1 and MMP-3 expression, the natural resorption process of HD and disc degeneration may vary with the patients’ age. In young human IVD, down-regulation or inhibition of MMP-3 may prevent induction of disc degeneration and disc herniation. In the case of old patients, artificial MMP-3 up-regulation may contribute to the natural resorption of HD by inducing disc degradation.

Conclusion
MCP-1 and MMP-3 expression in mouse IVD showed age-related decreases. The response in inflammation in IVD also displayed age-related changes. Therefore, disc degeneration may vary with the patients’ age and targeting MMP-3 may be a possible future therapeutic strategy for disc degeneration.

Acknowledgement
We thank professor Lynn M Matrisian at the department of cancer biology, Vanderbilt University Medical Center, Nashville TN, for providing MMP-3-deficient mice.