INTRODUCTION: Degenerated intervertebral discs (IVD) are typically accompanied by changes in biochemical properties, such as the loss of proteoglycan. Recent studies suggested these changes might be induced by matrix-degrading enzymes, such as the matrix metalloproteinases (MMPs) and ADAMTS [1, 2], after stimulation by proinflammatory cytokines (e.g., IL-1β and TNF-α).

Proteinase-activated receptor 2 (PAR2) is one of a unique subfamily of G protein-coupled receptors, which is known to be activated by several serine proteases (e.g., trypsin) [3, 4]. The activation of PAR2, which is induced by the exposure of the tethered ligand at the N-terminus by protease cleavage, has been reported to lead to inflammatory reaction and results in upregulation of cytokines in several types of cells [4]. Importantly, PAR2 expression itself is also upregulated by proinflammatory cytokines in an autocrine and/or paracrine manner [5, 6]. Thereby, PAR2 is considered to play a role in the regulation of inflammatory reactions and contributes to the pathophysiological condition of several inflammatory diseases in the joint, skin, airway, etc. [4]. Although PAR2 is known to be expressed in a wide range of tissues, including articular cartilage [6], the expression of PAR2 in the IVD remains unknown.

We hypothesized that PAR2 is expressed in IVD cells and is involved in the regulatory mechanism of IVD degeneration. The purpose of this initial study was to examine (1) the expression of PAR2 in rat IVD cells in vitro, (2) the effect of IL-1β on the expression of PAR2 and (3) the spatial distribution of PAR2 on rat IVD tissues in vivo.

MATERIALS AND METHODS:
Cell Preparation: Lumbar IVDs were obtained from Sprague-Dawley rat spines (age: 12 weeks, male, n = 10). The anulus fibrosus (AF) was carefully dissected from discs at six consecutive levels (L1/2 to L6/7). The AF cells were isolated by sequential enzyme digestion and cultured in monolayer at 4x10^4 cells/ml (DMEM/F12 + 10% FBS) [7].

Cell Culture: After seven days of pre-culture, the cells were cultured in serum-free medium (SFM) for 24 hours, and then cultured for an additional 48 hours in the presence or absence of recombinant human interleukin-1β (rhIL-1β; 1, 10 ng/ml) in DMEM/F12 + 0.3% FBS.

Western Blot: AF cell layers were lysed with RIPA buffer, including protease inhibitors, overnight at 4°C. Proteins (20 µg per well) were separated by SDS-PAGE (7.5% acrylamide) under reducing conditions, followed by Western blotting using a mouse monoclonal anti-human PAR2 antibody (SAM11: N-terminal of human PAR2 receptor, Santa Cruz). The AF cells were exposed to IL-1β (1, 10 ng/ml) for 48 hours and then harvested for Western blotting. The PAR2 protein expression was analyzed using a 55 kDa band.

Histological Analysis: Rat lumbar spines (L1 to L6) were removed and fixed in 4% paraformaldehyde followed by decalcification in 30% ethylenediaminetetra-acetic acid (EDTA) for 28 days. The samples were then embedded in paraffin. Sagittal sections (5 µm) were stained with Safranin-O and hematoxylin and eosin (H&E). Serial sections adjacent to the sections stained with Safranin-O/H-E were used for immunohistochemical analysis.

Immunohistochemistry: AF cells were cultured in monolayer on a chamber slide for seven days. The cells were then fixed in 100% methanol, permeabilized in 0.1% Triton-X. The cells were incubated with anti-PAR2 antibody (SAM11) overnight at 4°C, and then a secondary antibody, Alexa 488-conjugated anti-mouse IgG, was applied. The nuclei were stained with propidium iodide. Samples were imaged by confocal microscopy. Mouse IgG was used as an isotype control.

Rat IVD tissues were also stained using a protocol similar to the one described above. The coloration was performed by reaction with the immuno-peroxidase polymer using a Histofine® system (Nichirei). Rat skin was used for a positive control.

RESULTS:
PAR2 expression on cultured IVD cells: In IVD cells cultured in monolayer, immunoreactivity to the anti-PAR2 antibody was clearly identified. Confocal images revealed that the PAR2 immunoreactivity was prominently found in granules in the cytoplasm of IVD cells as previously reported [8] (Fig. 1). The localization of PAR2 immunoreactivity did not differ in the control and IL-1β (1, 10 ng/ml) treated cells. No immunoreactivity was found in the cytoplasm of the isotype control.

Western blotting: A single protein band with a molecular mass of about 55 kDa, which was consistent with the previous report of PAR2 [9], was confirmed. It is noteworthy that the treatment with IL-1β (1, 10 ng/ml) significantly increased the amount of PAR2 protein compared to the control (IL-1β: 0 ng/ml).

Distribution of PAR2 immunostaining in rat IVD tissues: PAR2 immunoreactive cells were found in the AF and NP tissues in both the anterior and posterior portion of IVDs (Fig. 3). PAR2 immunoreactivity was found in fibrochondrocytes that were aligned longitudinally between the collagen fiber bundles in the outer AF (Fig. 3A, D). However, immunoreactive cells were fewer in the inner AF. Smaller chondrocyte-like cells in the NP were positively stained (Fig. 3C). In the rat, the positive control (skin) showed a staining pattern similar to that in human skin [10] (data not shown).

DISCUSSION: We have demonstrated for the first time that PAR2 is present in rat IVD cells and NP and AF tissues. Importantly, our in vitro experiment showed that PAR2 expression was upregulated by IL-1β stimulation. These results raise questions on the biological role of PAR2 in the cytokine-mediated metabolic cascade in the IVD. Because PAR2 activation is known to trigger metalloprotease and cytokine production [4], it may be possible that PAR2 is involved in the mechanism of disc degeneration. Further studies on the expression of PAR2 and its regulatory mechanism in human degenerated discs need to be performed.