Type II Collagen Gene Expression of Intervertebral Disc in Ovariectomized Rats

Masanori Kato1, Nobuyuki Fujita1, Naobumi Hosogane1, Hironari Takaishi1, Jiro Takito1, Yoshiakı Toyama1, Kazuhiro Chiba1, Yoshiaki Toyama1, Kazuhiro Chiba1

1Orthopaedic Surgery, Keio University School of Medicine, Tokyo, Japan; 2National Hospital Organization Murayama Medical Center, Tokyo, Japan; 3Musculoskeletal Reconstruction and Regeneration Surgery, Keio University School of Medicine, Tokyo, Japan

Introduction: The intervertebral disc (IVD), which consists of water-rich nucleus pulposus (NP) and lamellar annulus fibrosus (AF), degenerates with age. IVD cells are able to maintain expression of type II collagen and proteoglycans as the main components of cartilaginous matrix. Estrogen deficiency in postmenopausal state closely associates with the development of osteoporosis and may induce vertebral fractures. Although it has been reported that the IVD in ovariectomized (OVX) rats showed greater degeneration than controls, the degenerative process is not fully understood (1). The purpose of this study is to investigate whether and how estrogen is involved in the degenerative process of the IVD. We examined whether estrogens stimulate type II collagen gene (Col2a1) expression in IVD cells, and whether transcriptional activity of Col2a1 is associated with a distinct estrogen receptor (ER)-dependent response mechanism.

Materials and Methods: Animals

Eight-week-old female Wistar rats were OVX or sham operated. We sacrificed the rats and collected the NP and AF from the tail IVD at 1 week, 2 weeks, 3 months, 7 months, and 1 year after the operation. The L5-L6 spinal unit was fixed in 10% neutral buffered formalin, decalcified by EDTA, paraffin-embedded, and sectioned to a 5μm thickness. The sections were stained with hematoxylin and cosin.

Real-time RT-PCR assay

The first strand cDNA was synthesized from total RNA of NP and AF of OVX or sham operated rats. Subsequent PCR amplification was carried out using a SYBR Premix Ex Taq (Takara) in a Light Cycler Quick System (Roche).

Cellular localization of Estrogen Receptors

AF cells were obtained from the isolated rat AF by digestion with 0.02% collagenase (Roche) and 0.4% pronase (Serva). AF cells plated on a glass coverslip were cultured in DMEM/F12 with 10% FCS. For immunofluorescence, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% NP-40 in PBS and incubated with primary antibody ERα (Santa Cruz), ERβ (Upstate). Also, rat AF cells were transfected with a plasmid expressing human ERα tagged with red-fluorescent protein (a kind gift from Dr. McDonnell, Duke University) using FUGENE HD (Roche). 17β estradiol (E2) (10⁻⁷ M), ICI182780 (10⁻⁷ M) or vehicle (ethanol) was added to the culture medium, and cells were processed for immunofluorescence for exogenous ERα with confocal microscopy.

Luciferase Reporter Gene Assay

Dual Luciferase Reporter Assay System (Promega) was used. Briefly, the COS-7 cells were co-transfected with a luciferase reporter plasmid containing col2a1 promoter element (2) including the Sox9 binding domain and 20-100ng of the ERα or ERβ expression vector and the rERα-(1–535) expression vector, DN-ERα, at the indicated amounts (0–100ng) in the presence or absence of 10⁻⁷ M E2 (3). All assays were performed in the presence of 2 g of pRL-TK, a Renilla luciferase reporter plasmid, as an internal control.

Results: Histological observations at 7 months after the surgery indicated that the cell number of the NP and AF decreased significantly in the OVX rats than that in the sham rats. Lamellar structure of the AF was disorganized in the OVX rats. RT-PCR detected the expression of ERα and ERβ, in both the NP and AF. The analysis of immunofluorescence using confocal microscope revealed that ERα and ERβ were localized in the nucleus of AF cells. In AF cells, exogenously transfected DsRed-tagged ERα tended to aggregate in the nucleus by E2 treatment and disperse by estrogen antagonist, ICI182780, administration. Real-time RT-PCR indicated that the expression level of col2a1 of the AF and NP in the OVX rats was approximately 0.4 times lower (P<0.05) than that in the sham rats at 2 weeks and 7 months after the surgery. In COS-7 cells transfected with only ERα but not with ERβ, relative luciferase activity of col2a1 was increased by up to 3 times in response to E2. On the other hand, DN-ERα inhibited the ERα-mediated activation of the col2a1 reporter activity.

Discussion: IVD degeneration is associated with loss of matrix molecules such as type II collagen, which leads to a change in the biochemical properties of the IVD. This study provides novel information on the expression of ERα and ERβ in rat IVD cells, suggesting a possible link between OVX and IVD degeneration. In classical ER signaling, the binding of E2 to ER initiates a cascade of events leading to strong ER dimerization, binding to estrogen response elements in the regulatory regions of target genes, and gene transcription. In addition to nuclear-initiated genomic responses, estrogen receptors have the ability to facilitate rapid, membrane-initiated, estrogen-triggered signaling cascades via a plasma membrane-associated form of the receptor. Decreased col2a1 mRNA expression and histological evidence of degeneration in the IVDs of the OVX rats suggest that menopause is one of the factors inducing disc degeneration and that col2a1 may be involved in the pathway as an estrogen-responsive gene in the osteoporotic spine. Our understanding of the ER-mediated pathway at the molecular level will provide insight against the controversies in estrogen therapy for postmenopausal women with degenerative disc disease and osteoporosis.