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Human articular chondrocytes exhibit sexual dimorphism in their responses to 17β-estradiol[1]

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Summary

Objective: The higher incidence of osteoarthritis in females suggests that there may be intrinsic sex-specific differences in human articular chondrocytes. 17β-Estradiol (E_2) regulates rat growth plate chondrocytes through traditional nuclear receptor mechanisms, but only female cells exhibit rapid membrane-associated effects mediated through protein kinase C (PKC) alpha. Here we demonstrate sexual dimorphism in the physiological response of human articular chondrocytes to E_2.

Methods: Articular chondrocytes were obtained at the time of autopsy from three male and three female donors between 16 and 39 years of age. Second passage cultures were treated with E_2 for 24 h to assess the effects of the hormone on [^{3}H]-thymidine incorporation, [^{35}S]-sulfate incorporation, and alkaline phosphatase specific activity. In addition, the chondrocytes were treated for 3, 9, 90 or 270 min and PKC specific activity was determined.

Results: All chondrocytes were positive for aggrecan and estrogen receptor alpha mRNAs but were negative for type II collagen mRNA. Only cells from female donors responded to E_2. DNA synthesis, sulfate incorporation and alkaline phosphatase activity were increased. E_2 caused a rapid increase in PKC activity in the female cells within 9 min that was maximal at 90 min. Treatment with the PKC inhibitor chelerythrine blocked these effects.

Conclusions: These results provide the first definitive evidence that normal human cells exhibit an intrinsic sex-specific response to E_2 and suggest that sexual dimorphism may be an important variable in assessing the pathways that modulate cell behavior.

Key words: Sex-specificity, Articular chondrocytes, PKC, Estrogen, Rapid actions.

Introduction

Epidemiological studies show sex-specific differences in both prevalence and severity of osteoarthritis[1–4]. Men exhibit higher prevalence of osteoarthritis before age 50, while women have a higher prevalence after age 50. In addition, postmenopausal women are more likely to have a general form of the disease that affects multiple joints with greater severity. This epidemiologic and pathologic gap between the sexes continues to increase with advancing age. Attempts to correlate biomechanical differences between males and females with the incidence and prevalence of osteoarthritis have failed to identify a causative factor, suggesting that there may be innate differences at the cellular level that contribute to disease severity.

One possibility is that sensitivity to estrogen plays a role. The presence of estrogen receptors (ER) alpha and beta in chondrocytes supports clinical observations that articular cartilage is an estrogen-sensitive tissue[5–8]. The parameters by which estrogen modulates chondrocyte behavior are not well understood, however. Human clinical studies using hormone replacement therapy have failed to show a consistent correlation between serum estradiol levels and development or progression of osteoarthritis[9–11]. Animal models have likewise shown differing results depending on the model[12–16].

Studies examining effects of 17β-estradiol (E_2) on growth plates of mice and rats in organ culture indicate that responses to the hormone differ in a sex-dependent manner[17], suggesting that the sexual dimorphism is at the cellular level. ERα and ERβ are present in rat growth plate chondrocytes[18], but female cells possess more high affinity

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receptors than male cells[19]. In addition, there are marked differences in physiological responses of male and female rat growth plate chondrocytes to E_2[20]. Female cells demonstrated decreased proliferation and increased alkaline phosphatase activity, indicating that E_2 promotes differentiation. E_2 also affected extracellular matrix production, resulting in increased synthesis of both collagen and proteoglycan. These effects were not seen in male cells or with 17α-estradiol.

Stereospecificity of the response to estrogen suggests a receptor-mediated mechanism. E_2-treatment increases membrane fluidity and phospholipid metabolism in female cells[21], and also results in rapid elevation of protein kinase C (PKC) activity[22]. The increase in PKC is mediated through G-proteins and phospholipase C[23]. A similar increase in PKC activity was observed when E_2 was conjugated with bovine serum albumin to prevent diffusion through the plasma membrane[24], thereby inhibiting interaction with cytosolic ERs. Whereas tamoxifen blocks E_2 activation of PKC in growth plate chondrocytes, neither the ER agonist diethylstilbestrol nor the ER antagonist ICI 182780 has an effect[18], suggesting that E_2 regulates chondrocytes through mechanisms other than those traditionally associated with nuclear receptors. E_2 also activates PKC in human colon cancer cells only from females[25], and activates PKC in female rat distal colon by an ICI 182780-insensitive mechanism[26].

Our goal was to determine if sexual dimorphism in response to estrogen is a feature of human articular chondrocytes. Cells from multiple donors were examined to verify that differences in response were due to genetic sex and not normal human variation. We first characterized the human articular chondrocytes to ensure they were phenotypically chondrocytes, and then examined their response to E_2 by measuring changes in proliferation, differentiation and matrix deposition. Finally, we investigated the potential role of PKC signaling in E_2 stimulation of human articular chondrocytes.

Materials and methods

HUMAN ARTICULAR CHONDROCYTE ISOLATION AND CELL CULTURE

Articular cartilage was isolated from the femoral condyles and tibial plateaus of human donors made available due to autopsy. Donors had no known history of joint disease and histological analysis was performed at the time of isolation to confirm the absence of any pathologies. The articular cartilage was cut into small pieces and washed twice for 20 min with Hank's balanced salt solution (HBSS) containing 1% penicillin and streptomycin. The washed cartilage was digested for 1 h with 0.25% trypsin-1 mM ethylene diamine tetraacetic acid (EDTA), followed by treatment with 0.2% collagenase for 3 h. All enzymes were prepared in HBSS. The digested suspension was passed though a 40 μm mesh sieve and centrifuged at 2000 rpm for 10 min. The supernatant was removed and the chondrocytes were resuspended in full media containing 88% Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-ascorbic acid[27].

Isolated primary chondrocytes were plated on T75 flasks, grown to confluence, and harvested using 0.25% trypsin-1 mM EDTA. The cell suspension was centrifuged at 2000 rpm for 10 min, the supernatant removed, and the cells resuspended in cold DMEM, 20% FBS, 1% penicillin/streptomycin, and 5% dimethyl sulfoxide. The chondrocytes were then frozen at $-80\,^{\circ}C$ and shipped overnight from La Jolla, CA to Atlanta, GA. The chondrocytes were thawed, centrifuged, resuspended in full media and plated on T75 flasks. These cultures were grown to confluence and passaged one time as above. Thus second passage human chondrocytes were used for all experiments. Confluent second passage cultures were also used to assess expression of chondrocyte phenotypic markers. It was necessary to expand the cells in culture in order to obtain sufficient numbers for each set of assays.

EXPRESSION OF AGGRECAN AND COLLAGEN

Articular chondrocytes dedifferentiate when expanded in monolayer cell culture[28,29]. Therefore, reverse transcription polymerase chain reaction (RT-PCR) was used to assess the phenotype of the second passage human chondrocytes by measuring the mRNA expression of aggrecan, collagen I, collagen II, and collagen X. Total RNA was extracted from the chondrocyte cultures with Trizol reagent. Lipophilic contaminants were removed by adding chloroform and centrifuging for 25 min at 4700 rpm. The aqueous phase was then washed with isopropyl alcohol to precipitate the RNA, and centrifuged for 20 min at 4700 rpm to form a pellet. The pellet was washed with cold 70% ethanol and centrifuged for 15 min at 4700 rpm. The supernatant was removed and the pellet allowed to air dry. The RNA was then dissolved in diethyl pyrocarbonate (DEPC) treated water and the purity and quantity was determined by UV spectrophotometry.

The total RNA sample from each donor was reverse transcribed using the First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) and the specific anti-sense primer for each mRNA of interest. The cDNA was amplified using the Fisher PCR kit (Fisher Scientific International, Hampton, NH) and the specific sense and anti-sense primers. PCR conditions for each cycle included a 30 s denaturation at $94\,^{\circ}C$, a 60 s annealing at $50-65\,^{\circ}C$ depending on the primer used, and a 30 s extension at $72\,^{\circ}C$. The PCR-amplified products were run on 5% polyacrylamide gels using a buffer consisting of 0.9 M Trizma® base (tris[hydroxymethyl]aminomethane), 0.9 M boric acid, and 20 mM EDTA. Primary human fibroblast RNA was used as the positive control for collagen I, and RNA extracted from rat growth plate cartilage was used as the positive control for collagen X. The negative control for the extracellular matrix components was human lymphocyte RNA.

The primer sequences and annealing temperatures for each marker were as follows. Aggrecan sense primer was 5′-TGA GGA GGG CTG GAA CAA GTA CC-3′ and anti-sense primer was 5′-GGA GGT GGT AAT TGC AGG GAA CA-3′ with an annealing temperature of $65\,^{\circ}C$. Collagen I sense primer was 5′-GTC AGG CTG GTG TGA TGG GA-3′ and anti-sense primer was 5′-AAC CTC TCT CGC CTC TTG CT-3′ with an annealing temperature of $59.8\,^{\circ}C$. Collagen II sense primer was 5′-CTG CTC GTC GCC GCT GTC CTT-3′ and anti-sense primer was 5′-AAG GGT CCC AGG TTC TCC ATC-3′ with an annealing temperature of $56.4\,^{\circ}C$. Collagen X sense primer was 5′-AGT CCT GGA CTC CAA CGA-3′ and anti-sense primer was 5′-TGG AAG ACC CCT CTC AC-3′ with an annealing temperature of $54.6\,^{\circ}C$.

ER EXPRESSION

RT-PCR for ERα was performed as above except RNA from human ovarian tissue was used as a positive control.