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Aromatase expression in human chondrocytes: An induction due to culture

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ABSTRACT

Objectives: Despite the high prevalence of osteoarthritis (OA) in postmenopausal women, a relationship between circulating estrogen levels and the development of OA has not been found. Therefore, the purpose of this study was to evaluate the expression and activity of aromatase, a key enzyme in local production of estrogens, in human OA cultured articular chondrocytes, and to determine the physiological relevance of this enzyme in cartilage.
Methods: Human OA articular chondrocytes were isolated and cultured. Local production of estradiol was measured after incubation with 100ng/ml testosterone for 8 and 24 h. Furthermore, chondrocytes were culture for 2 h, 48 h, 7 days or 15 days, or in alginate beads for 10 days. Aromatase, type II and X collagen, aggrecan, alkaline phosphatase, and Runx2 expression were evaluated in cartilage, freshly isolated chondrocytes and cultured chondrocytes.
Results: Aromatase was expressed and active in cultured human chondrocytes. Human cartilage, freshly isolated chondrocytes, and chondrocytes cultured for 2 h expressed an insignificant amount of aromatase; however, expression arose after 48 h of culture and remained increased thereafter. Aromatase expression was not related to estrogen deprivation and was inversely correlated with differentiation. Re-differentiation did not reduce its expression.
Conclusions: Aromatase presents an almost undetectable expression in human cartilage but is induced in cultured chondrocytes. Therefore, human cartilage might act as a mere target for estrogens rather than a producer, and researchers using cell expansion in culture for latter therapies should consider these changes in estrogen metabolism which may not be reverted after re-differentiation.

1. Introduction

Several studies have demonstrated that systemic estrogens are important in cartilage maintenance. Estrogens increase proteoglycan production and reduce cartilage destruction and pro-inflammatory mediators in chondrocytes [1]. Moreover, estrogen deficiency is related to cartilage damage and osteoarthritis (OA) development, since ovariectomized animals and menopausal women present increased cartilage turnover and greater OA prevalence [1,2]. Very recently, it has been reported that knee OA is associated with lower circulating levels of estradiol [3]. However, previous studies failed to find a correlation between OA progression [4], or found a correlation with incidence but not with prevalence of the disease [5] when a multivariable model was used. Regarding hormone replacement therapy, no effect on knee pain [6] or radiographic knee OA [7] was observed, and a systematic review found only some evidence of a protective effect of unopposed estrogen use for hip OA [8]. Therefore, despite certain evidence, there is not a clear relationship between circulating estrogen levels or exogenous estrogen administration and the development of OA [1,9,10], and it has been suggested that, during menopause, locally produced estrogens from androgens may play an important role in joint tissues protecting cartilage from damage [9].

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Aromatase is a key enzyme in locally produced estrogens, converting testosterone to estradiol and androstenedione to estrone. It is expressed in several tissues, such as ovary, placenta, breast, brain and adipose tissue [11]. Aromatase has also been detected in human bone; in fact, low aromatase expression has been found in OA bone [12]. In addition, the use of aromatase inhibitors during breast cancer therapy has been related to bone loss and an increased incidence of arthralgia [13,14]. However, only few studies have evaluated aromatase expression in cartilage or have investigated the biological importance of aromatase for this tissue.

Aromatase expression has been detected by *in situ* hybridization and PCR in the growth plate and cultured chondrocytes from rats [15–17], while its activity has been demonstrated in both articular and costochondral rat chondrocytes after being cultivated [16,17]. Regarding humans, aromatase has been detected by immunohistochemistry in human growth plate [18] and Sasano et al. detected aromatase by *in situ* hybridization in some chondrocytes of human articular cartilage [19]. Very recently, Schicht et al. showed aromatase expression in human cartilage [20].

The aim of this study was to evaluate aromatase expression and activity in human knee OA cultured chondrocytes and in OA cartilage samples to determine the physiological relevance of this enzyme in cartilage biology. In addition, since we observed an up-regulation of aromatase expression during culture, we also investigated the factors contributing to this effect.

2. Methods

2.1. Cell culture

Human tissues were obtained from FJD Biobank (IIS-Fundación Jiménez Díaz, Madrid). Samples originating from the articular cartilage of OA women over 65 years old who had undergone knee joint replacement surgery were used for this study, and written informed consent was obtained from all patients. The Clinical Research Ethics Committee of the Fundación Jiménez Díaz approved the protocol.

Chondrocytes were isolated by trypsin digestion (Lonza, Belgium) for 30 min at 37 °C followed by 1 mg/ml of collagenase type IV (Sigma–Aldrich, USA). 300.000 cells per well were seeded in 6-well plates and maintained at 37 °C and 5%CO_2. As the phenol red present in standard culture medium shows estrogenic effects [21], and the hormones present in FBS can interfere with estrogen metabolism, the culture medium used for chondrocyte isolation and maintenance was phenol red-free DMEM with 10% charcoal-stripped fetal bovine serum (FBS), containing 2 mM glutamine and 100 U/ml of penicillin and streptomycin (Lonza, Belgium).

For the time course of aromatase expression, freshly isolated chondrocytes were cultured as described above for 2 h, 48 h, 7 days, or 15 days in medium supplemented with either standard or charcoal-stripped FBS.

In addition, some chondrocytes were cultured in monolayer until confluence under standard conditions (DMEM with phenol red and standard FBS). Then, cells were harvested and either embedded in alginate beads at a concentration of 4×10^6 cells/ml as previously described [22] or plated in monolayer. Monolayer cultures and beads were maintained in culture for 10 days in phenol red-free DMEM supplemented with charcoal-stripped FBS.

2.2. Aromatase activity

Cells were grown for 2 to 3 weeks until confluence. After 18 h in serum-free conditions, chondrocytes were cultured in the presence or absence of 100ng/ml of testosterone (Sigma–Aldrich, USA) for 8 h or 24 h. Then, estradiol production was measured in culture medium using Estradiol Sensitive ELISA kit (DRG International, Germany) and RNA was isolated using Tripure isolation reagent (Roche, USA) according to the manufacturer's protocol.

2.3. Gene expression analysis

For RNA isolation, cultured cells were lysed using Tripure reagent. Alginate beads were dissolved (sodium citrate 55 mM + NaCl 150 mM) and RNA from cells was isolated using NucleoSpin RNA kit (Macherey-Nagel, Germany). For total cartilage, tissue was ground into a powder and mixed with Tripure reagent. RNA was isolated according to the manufacturer's protocol and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). 40ng of cDNA were used to quantify aromatase, type X collagen (Col10), Runx2, and alkaline phosphatase (ALP) gene expression by Real Time PCR using Aromatase TaqMan gene expression assay and human HPRT as the endogenous control (Applied Biosystems, USA). To assess cell differentiation, type II collagen (H_COL2A1_1) (Col2), aggrecan (H_ACAN_1) and GAPDH (H_GAPDH_1) gene expressions were measured by Real Time PCR using KiCqStart SYBR Green Primers (Sigma Life Sciences). Relative gene expression was quantified using the $\Delta\Delta$Ct method, and all data are presented as relative expression to freshly isolated chondrocytes or non-stimulated cells.

2.4. Immunohistochemistry

Human cartilage samples were fixed in 4% paraformaldehyde for 24 h, washed with 70% ethanol and then embedded in paraffin wax. Sections of 5 μm were incubated with rabbit polyclonal anti-estrogen receptor (ER)α or anti-ERβ (sc-542 and sc-8974 respectively, Santa Cruz Biotechnology Inc, USA) followed by a biotinylated anti-rabbit IgG and visualized using horseradish peroxidase/AB complex (DakoCytomation, USA). An IgG isotype was used as a negative control.

2.5. Statistical analysis

For all the experiments, at least 4 independent samples from different patients were used. Data were analyzed using Mann–Whitney and Spearman correlation tests with Prism software (v5.01, GraphPad sotfware, Inc). Results were expressed as median ± interquartile range. $p < 0.05$ was considered statistically significant.

3. Results

Human OA chondrocytes cultured until confluence were able to produce significant amounts of estradiol after 8 and 24 h of exposure to testosterone (Fig. 1A). This activity was due to the expression of a functional aromatase, which was expressed in the chondrocytes even in the absence of this androgen and was not modulated by it (Fig. 1B). Interestingly, the greatest amount of estradiol was accumulated during the first 8 h of treatment, while in the following 16 h the concentration of estradiol was only slightly increased.

Aromatase mRNA in human healthy ($n = 3$) and OA cartilage ($n = 7$) was detected at very high Ct, indicating that the cDNA of aromatase was very low and more PCR cycles were needed to detect the amplified product. In fact, 66% of the samples showed a Ct above 38, representing an almost undetecTable expression. Freshly isolated OA chondrocytes, as well as chondrocytes after 2 h of culture, showed a similar expression. However, aromatase expression arose in cultured chondrocytes after 48 h and remained increased thereafter (Fig. 2A).