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Expression of genes for estrogen receptors α and β in human articular chondrocytes

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Summary

Objective: To investigate the gene expression of estrogen receptor (ER) α and ERβ in human articular chondrocytes.

Methods: 16 articular cartilage specimens were obtained from 15 patients during surgery. Three of the specimens were from men and 13 from women; three from hip joints and 13 from knee joints; four were normal and 12 showed osteoarthritic cartilage. Total RNA was extracted from the articular chondrocytes and the expression of both ERα and ERβ genes was investigated by the reverse transcription-polymerase chain reaction (RT-PCR) method.

Results: Gene expressions of ERα were detected in all specimens and those of ERβ were found in 15 specimens by the RT-PCR method. There was a significant correlation between the amounts of ERα and ERβ. Expression levels of both genes were significantly higher in men than in women. There were no significant differences in the expression levels of both ER genes between the hip and knee joint sites, nor between normal and osteoarthritic tissues.

Conclusion: This study is to our knowledge the first to demonstrate the gene expression of both ERα and ERβ in human articular chondrocytes. Since there are some functional differences between the two receptors, the effects of estrogen on cartilage metabolism should be elucidated by two different receptor mechanisms. © 1999 OsteoArthritis Research Society International

Key words: Articular chondrocytes, Estrogen receptor α, Estrogen receptor β, Osteoarthritis.

Introduction

There are significant sex differences regarding the prevalence and clinical manifestations of osteoarthritis (OA). OA is more likely to occur in women, especially postmenopausal, and the symptoms are more severe than in men. Therefore, the potential role of estrogens in the pathogenesis of OA has been suggested.[1–7]

As a possible mechanism of estrogen action, it has been suggested that estrogen affects articular cartilage metabolism directly via an estrogen receptor (ER) in chondrocytes. An ER in chondrocytes has been detected by radioligand assay,[8–14] immunoprecipitation,[10,14–17] autoradiography,[13,15,18] and in-situ hybridization.[16] Some studies, however, have failed to show the presence of an ER in chondrocytes by radioligand assay.[19,20] Most of the previous studies were carried out using cultured chondrocytes from animal or human epiphyseal cartilages, whereas the presence of an ER in human articular cartilage has been confirmed only in one report, by Tsai *et al*.[11]

Recently, it has become evident that there is not only a classical ER (ERα), but also a novel ER (ERβ).[21,22] The tissue distribution of the two receptors was found to be different in rat and human fetuses.[23,24] In the human fetus, ERα mRNA is most abundant in the uterus, while high amounts of ERβ mRNA are present in the ovaries, testes, adrenals, and spleen.[24] The levels of transactivation are higher for ERα than for ERβ, although estradiol binds to both ERα and ERβ and their binding affinity is almost identical.[22,23] These observations suggest different organ-specific roles for the two receptors. Nillson *et al*.[17] demonstrated of ERβ immunoreactivity in human growth plate cartilage. Until now, it is not known what type of ER is expressed in human articular chondrocytes.

In this study, we investigated the expression of ERα and ERβ genes in human articular chondrocytes in relation to joint sites, sex, and osteoarthritic changes.

Materials and methods

SUBJECTS

Articular cartilage tissues from 16 joint sites of 15 patients (12 women and three men) aged from 39 to 85 (mean 70.5), were obtained during orthopedic surgery. Of the 16 specimens, three were from men and 13 from women. Three were from hip joints and 13 from knee joints. Of the 13 knee specimens, 12 were obtained during total knee replacement for knee OA, and one during above knee amputation for a malignant bone tumor of the distal femur, the former being categorized as osteoarthritic and the latter as normal. Three hips underwent endoprosthetic replacement for a femoral neck fracture, being categorized as having normal cartilage. As a result, four specimens were normal and 12 were osteoarthritic cartilage. Patient details

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Table I
Clinical information for each patient

Patient No.	Age (y)	Sex	Joint site	Disease	Cartilage status	Cartilage specimen
1	70	f	Knee	OA	OA	Fem and tib
2	75	f	Knee	OA	OA	Fem and tib
3	67	f	Knee	OA	OA	Fem and tib
4	77	f	Knee	OA	OA	Fem and tib
5	77	f	Knee	OA	OA	Fem and tib
6R	63	f	Knee	OA	OA	Fem and tib
6L	63	f	Knee	OA	OA	Fem and tib
7	71	f	Knee	OA	OA	Fem and tib
8	73	f	Knee	OA	OA	Fem and tib
9	72	f	Knee	OA	OA	Fem and tib
10	80	m	Knee	OA	OA	Fem and tib
11	64	m	Knee	OA	OA	Fem and tib
12	84	f	Hip	Femoral neck fracture	Normal	Femoral head
13	85	f	Hip	Femoral neck fracture	Normal	Femoral head
14	61	f	Hip	Femoral neck fracture	Normal	Femoral head
15	39	m	Knee	Bone tumor	Normal	Fem and tib

OA, osteoarthritis; fem, femoral condyle; tib, tibial plateau.

Table II
Sequences of primers

Gene		Sequence	Size (bp)	Reference
ERα	Sense	5′-GACCGAAGAGGAGGGAGAA-3′	460	26
	Anti-sense	5′-CCAAGAGCAAGTTAGGAGCAA-3′		
ERβ	Sense	5′-TAGTGGTCCATCGCCAGTTAT-3′	323	27
	Anti-sense	5′-GGGAGCCACACTTCACCAT-3′		
	Sense nested	5′-CGGAACCTCAAAAGAGTCCCTGG-3′		
	Anti-sense nested	5′-CCGAAGTCGGCAGGCCTGGCAGC-3′		
GAPDH	Sence	5′-GAGTCAACGGATTTGGTCGT-3′	156	28
	Anti-sense	5′-GGTGCCATGGAATTTGCCAT-3′		

are shown in Table I. During the operation, the cartilage of the femoral condyle and tibial plateau (knee), or of the femoral head (hip), was carefully sliced off, not including the calcified cartilage layer or subchondral bone, and immediately preserved in liquid nitrogen. All women were postmenopausal and had never received hormonal replacement therapy. None of them received an intra-articular injection within one month before surgery.

RNA EXTRACTION

Total RNA was isolated by the acid guanidinium isothiocyanate-phenol-chloroform method[25] with slight modification. Briefly, the sliced cartilage specimen, preserved in liquid nitrogen, was homogenized in 10 volumes of 4 M guanidinium isothiocyanate/50 mM Tris-HCl (pH 7.5)/10 mM EDTA/2% sodium dodecyl sarcosinate/150 mm 2-mercaptoethanol using a homogenizer (HG-30, HITACHI, Tokyo, Japan). Two volumes of 0.1 M sodium acetate buffer (pH 5.2)-saturated phenol/chloroform were then added and the mixture was vigorously shaken at 60°C. The aqueous phase was recovered, then an equal volume of 0.1 M sodium acetate buffer-saturated phenol/chloroform was added to it, and the mixture was shaken at room temperature until the aqueous phase became clear. The aqueous phase was recovered, an equal volume of chloroform was added, and the mixture was shaken again. Then two volumes of absolute ethanol were added to the recovered aqueous phase and the mixture was allowed to stand for 18 h at −20°C. After centrifugation (10,000 **g**, 20 min at 4°C), the pellet was dissolved in 1 ml of 0.1 M Tris-HCl (pH 7.5)/50 mM NaCl/10 mM EDTA/0.2% SDS containing proteinase K (100 μg/ml) and incubated for 1 h at 37°C. The solution was treated with saturated phenol/chloroform, following ethanol precipitation. After centrifugation (15,000 **g**, 20 min at 4°C), the pellet was dissolved in 3 mM sodium acetate buffer (pH 5.2).

To remove genomic DNA contamination, each sample was treated with DNase I (RNase-free, Nippongene, Toyama, Japan) in the presence of RNase inhibitor (Wako pure chemical, Osaka, Japan) for 1 h at 37°C, followed by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. The pellet was dissolved in 3 mM sodium acetate buffer (pH 5.2) again and quantified spectrophotometrically at 260 nm.

RT-PCR (REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION)

RT-PCR was performed using an RNA PCR kit (TAKARA SHUZO, Otsu, Japan). Briefly, reverse transcription of RNA into cDNA was performed by incubating 2 μg of total RNA with AMV reverse transcriptase, 9 mer random primers, dNTP, and RNase inhibitor at 30°C for 10 min, followed by

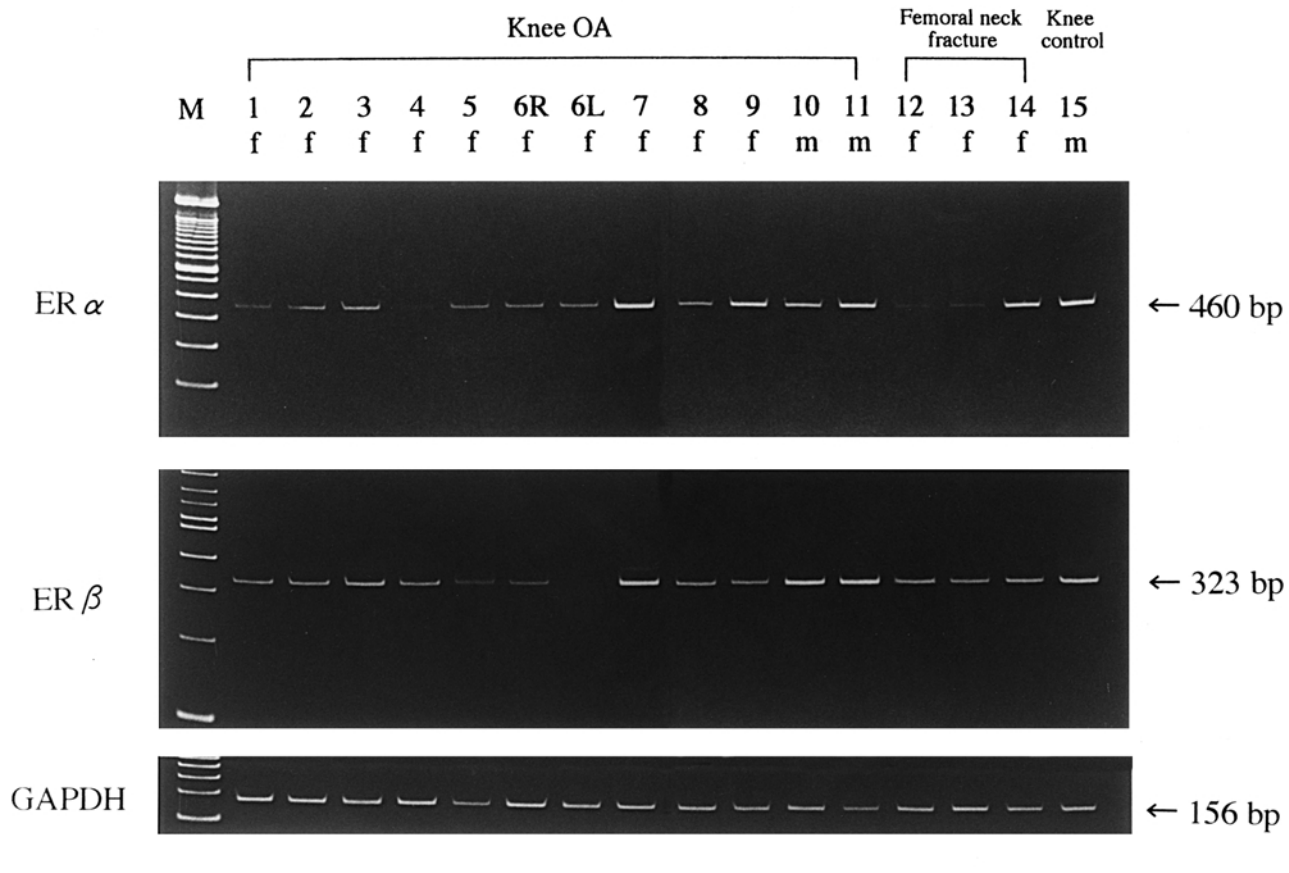

Fig. 1. RT-PCR amplification of ERα and ERβ mRNA in human articular chondrocytes. The photograph of polyacrylamide gel electrophoresis shows the gene expression of ERα, ERβ, and GAPDH as single bands of expected sizes in each cartilage specimen.

42°C for 30 min. One microliter (50 ng) of each cDNA sample from the above extractions was amplified by PCR in a total volume of 100 μl using 2.5 units of Ex Taq polymerase with the accompanying buffer, 200 μm dNTP mixture (TAKARA SHUZO), and 0.2 μm each of specific oligonucleotide primers. The oligonucleotide primers were prepared according to the published sequence of the human gene for ERα,[26] and previous reports for ERβ[27] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[28] (Table II). PCR was performed using a GeneAmp System 2400 (Perkin Elmer). The PCR condition for ERα was 35 cycles (94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min). For ERβ, nested PCR was performed. After 20 cycles (94°C for 30 sec, 53°C for 30 sec, 72°C for 1 min), 5 μl of the first PCR product was amplified again with nested primers in the same PCR condition for 20 cycles. The PCR condition for GAPDH was 20 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec). As a negative control, the above PCR procedure was performed simultaneously with an RNA sample that had not been reverse-transcribed and with distilled water, instead of cDNA. The amplified products were separated by electrophoresis using 6% polyacrylamide gels. To determine the size of the fragments, a 100 bp DNA ladder (Gibco BRL, MD, U.S.A.) was used as the marker. The gels were stained using ethidium bromide (0.5 μg/ml) and the cDNA bands were photographed under UV illumination. The fluorescent intensity of the expected bands was semi-quantified by computer analysis, i.e., the photographs were scanned and analysed using Adobe Photoshop software (Adobe Systems Inc., CA, U.S.A.). Results were normalized for GAPDH mRNA and expressed in arbitrary units.

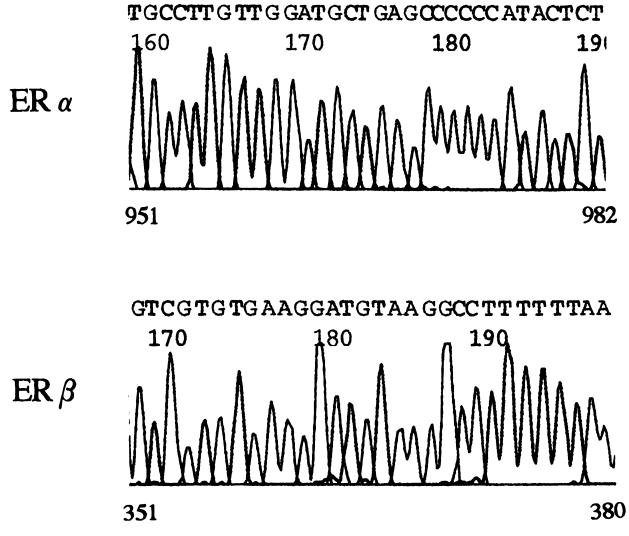

Fig. 2. Direct sequencing of ERα and ERβ PCR amplified products. The nucleotide sequences of the amplified cDNA fragments for both ERα and ERβ were consistent with those of ERα and ERβ, respectively.

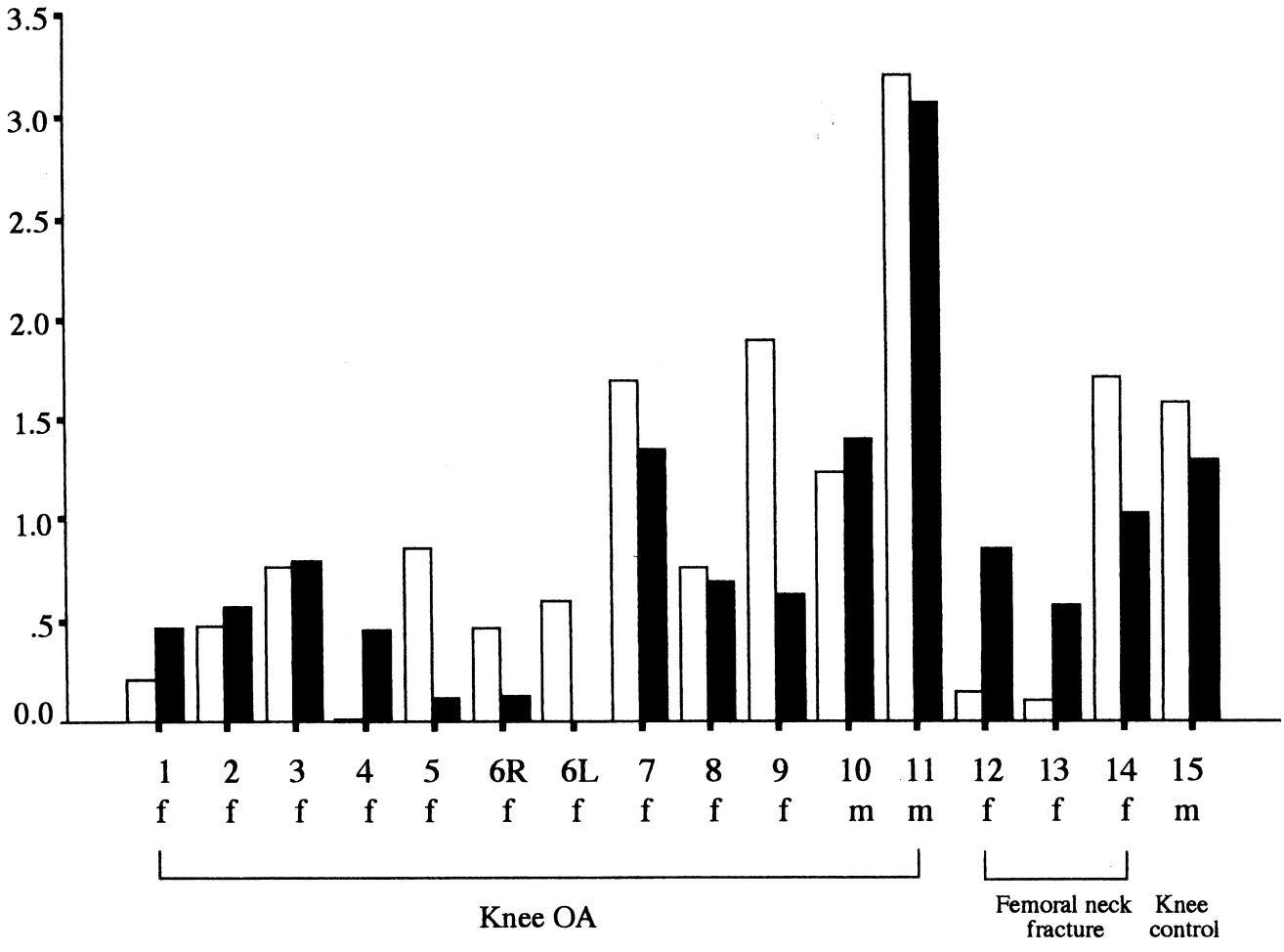

Fig. 3. Relative levels of ERα and ERβ gene expression in human articular cartilage. The calculated amounts of PCR products of ERα (□) and ERβ (■) standardized by that of GAPDH are shown for 16 cartilage specimens.

DIRECT SEQUENCING OF PCR PRODUCTS FOR ERα AND ERβ

The ERα and ERβ cDNA fragments, extracted from 6% polyacrylamide gels, were sequenced using an ABI 310 sequencer (Perkin Elmer, CA, U.S.A.) according to the manufacturer's protocol.

STATISTICAL ANALYSIS

We used a Student's *t*-test to compare the levels of gene expression between two groups, as well as linear regression analyses.

Results

In each PCR procedure, the amount of PCR product increased for up to 40 cycles for ERα and GAPDH, and for up to 20 cycles in nested PCR for ERβ, and increased almost linearly as a function of the amount of total RNA used (data not shown). No PCR product was observed in the procedures with the RNA sample that had not been reverse-transcribed or with distilled water, instead of cDNA, showing that the amplified product was mRNA-specific and that there was no genomic DNA contamination.

Figure 1 is a photograph of the polyacrylamide gel electrophoresis showing the expression of ERα, ERβ, and GAPDH as single bands of expected sizes for each cartilage specimen. Expression of the ERα gene was detected in all 16 specimens and that of the ERβ gene was detected in 15 by this method. The nucleotide sequences of these amplified ERα and ERβ cDNA fragments were consistent with those of ERα and ERβ, respectively[26,27] (Fig. 2).

The amount of the PCR products for each specimen standardized by that of GAPDH are shown in Fig. 3. Figure 4 shows the correlation of the amount of gene expression between ERα and ERβ in each specimen. The expression of both ER genes was correlated with each other using linear regression (ERα=0.197+0.938 ERβ, r^2=0.641, P=0.0002).

The amounts of gene expression were compared between various categories: men and women, joint sites, and normal and osteoarthritic cartilage. Gene expression levels were significantly higher in men than in women for ERα (P=0.015) and ERβ (P=0.001) (Fig. 5). There were no significant differences for either ER type between hip and knee, or between normal and osteoarthritic cartilage.

The correlation between the amount of each ER and age was analysed using a linear regression model. Although there was no significant relationship between the patients'

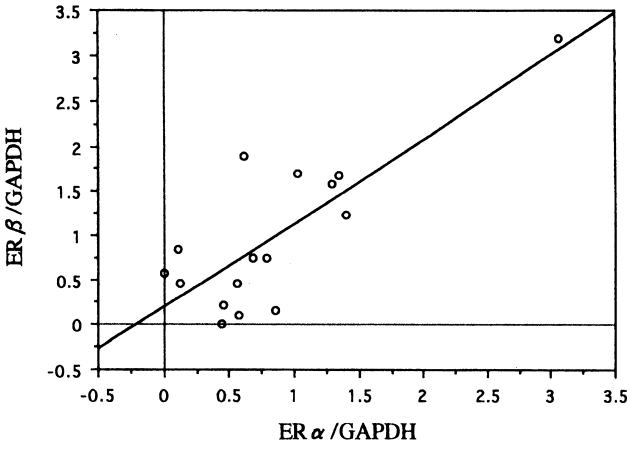

Fig. 4. Correlation of gene expression levels between ERα and ERβ. The expression of both ERs were correlated with each other (ERα=0.197+0.938 ERβ, r^2=0.641, *P*=0.0002).

age and ERβ gene expression (*P*=0.43), there was a mild tendency between the patients' age and ERα gene expression (*P*=0.09). In a separate analysis, the correlation between ER gene expression levels and age was analysed by sex, yielding again no significant relationship (data not shown).

Discussion

In this study we demonstrated the expression of both ERα and ERβ genes in human articular cartilage by the RT-PCR method. We detected ER mRNA in a fresh frozen cartilage specimen, indicating in-vivo ER expression in human articular chondrocytes. The levels of ERα and ERβ gene expression were correlated with each other. Men showed a significantly higher level of gene expression for both types of ERs than women. There were no apparent differences between joint sites, or between normal and osteoarthritic cartilage.

There has been an accumulating knowledge that estrogen modulates the metabolism of chondrocytes. Administration of estradiol worsened the erosive changes in meniscectomy-induced OA in rabbits, and this effect of estrogen was inhibited by tamoxifen, an estrogen antagonist.[29–31] Knee OA is induced by an intra-articular injection of estradiol in rabbits.[32] Cyclofenil diphenol, a non-steroidal weak estrogen, inhibited the synthesis of proteoglycan and hyaluronate in primary cultures of chondrocytes from the Swarm rat chondrosarcoma.[33] Testosterone and estradiol synergized with IL-1 in the induction of IL-6 in human articular chondrocytes.[34] These results suggest that an overdose of estradiol has an adverse effect on the articular cartilage.

On the other hand, in a study by Corvol *et al.*,[35] estradiol had stimulatory effects on cartilage proteoglycan synthesis in cultured chondrocytes from rabbits, and the response of female-derived cells was twice that of male-derived cells. Estradiol as well as testosterone stimulated DNA synthesis of chondrocytes from rat epiphyseal cartilage.[36] Itagane *et al.*[37] noted that sex hormones such as estrogen, progesterone, and testosterone had no stimulatory effects on DNA synthesis in rabbit chondrocytes, but they have priming effects on the biological action of insulin-like growth factor-I which affects the metabolism of the cartilage. In a recent study by Morisset *et al.*,[38] estradiol inhibited COX (cyclooxygenase)-2 in basal conditions in primary cultures of bovine chondrocytes. The protective effects of estrogen on the mechanical property of articular cartilage in ovariectomized sheep were shown by Turner *et al.*[39]

These conflicting results regarding the effects of estrogen on articular cartilage may be due to the differences between human and animal species, epiphyseal and articular cartilage, and *in vivo* and *in vitro* studies. Assuming that ERα and ERβ are functionally different, the status of differential expression of two different ERs could partly explain the controversy surrounding the role of estrogen in

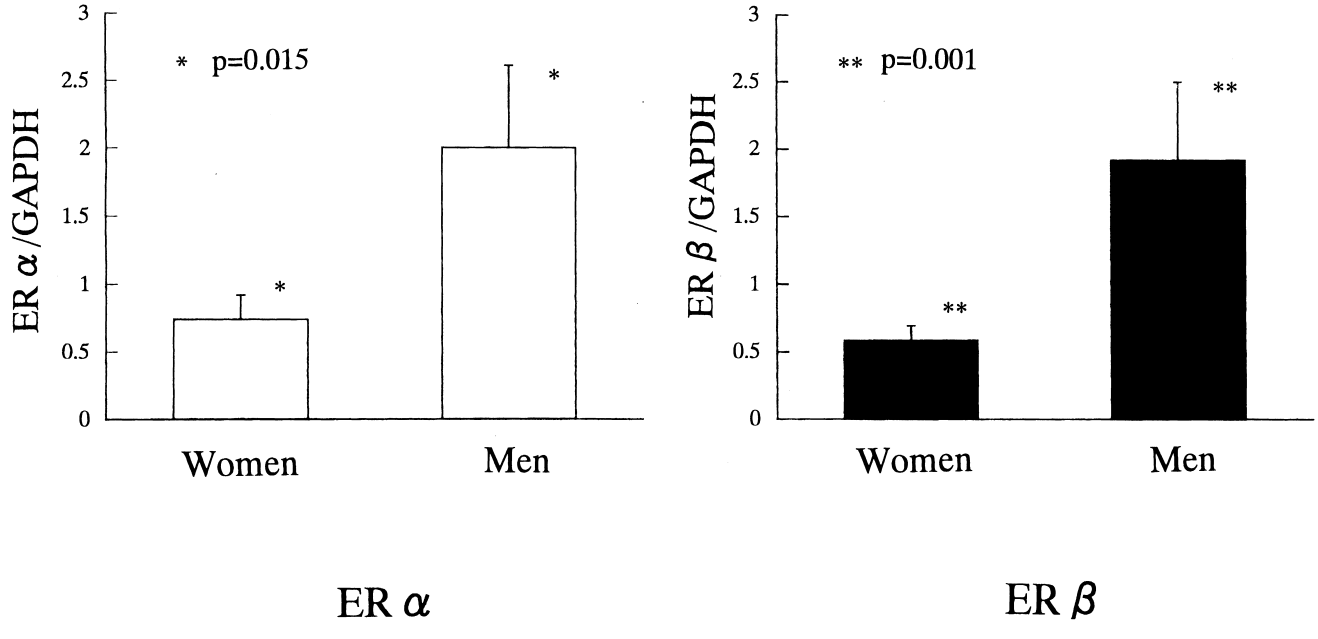

Fig. 5. Comparison of gene expression levels of both ERs between men (*N*=3) and women (*N*=13). For both ERα and ERβ, men had a higher expression level in amounts.

cartilage metabolism. Interestingly, ERα and ERβ are expressed differently during human osteoblast differentiation.[27] ERβ mRNA expression increased gradually during osteoblast culture, whereas ERα mRNA expression levels increased slightly at the beginning and soon remained constant, suggesting an additional functional role of ERβ to ERα in bone metabolism. Therefore, the functional role of the two ERs in chondrocyte metabolism should be addressed in future studies.

From a clinical point of view, it is easier to postulate that estrogens are beneficial to articular chondrocyte metabolism, because the age at onset of OA in women has been found to coincide with the perimenopausal period when serum estrogen levels rapidly decline.[1] In fact, some studies have shown that estrogen replacement therapy has protective effects for hip and knee OA.[40,41] The one paradoxical fact against the beneficial role of estrogen on cartilage is a high female to male prevalence ratio shown by epidemiological surveys of OA.[1] Estrogen levels are generally higher in women than in men, although sex steroid levels in both genders tend to converge in older age.[42] As shown in the present study, women have a lower level of ERα and ERβ, suggesting that women would be more prone to a fluctuation in serum estrogen levels. This might give a possible explanation for the apparent paradox.

In this study, however, the number of cartilage specimens were small, especially in the male and control subjects, and the histological findings presenting the degenerative status of cartilage were not evaluated. Therefore, the expression of both ERs in various joint sites in relation to age, sex, and cartilage status, and the events that follow estrogen binding to both ERs in articular chondrocytes need to be elucidated in further studies.

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